UNIVERSITY OF GLASGOW

IMMUNOLOGICAL STUDIES OF GONADOTROPHIC HORMONES

by

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Thesis submitted for the degree of
Ph.D. in the Faculty of Medicine.

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February, 1963.

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ACKNOWLEDGMENTS.

I owe my gratitude to Professor T. Symington for his kindness, and to my supervisor, Dr. A.D.T. Govan, for his great help, patience, guidance and continuous encouragement.

I am also indebted to Dr.J.R. Anderson, Pathology Department, Western Infirmary, for his advice on the serological techniques, and to Dr. B. Cruickshank, Pathology Department, Royal Infirmary, for his criticism and advice on the gel diffusion experiments.

I would like also to thank Mr. A. Fraser, Chief Technician, and the rest of the technical staff of the Research Department, Glasgow Royal Maternity Hospital, for their help and kindness.

Finally, I thank Miss K. Quinn for typing this Thesis.

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INTRODUCTION.

INTRODUCTION

Historical:

The idea of hormone inhibiting substances which circulate in the blood stream and are able to neutralise the effects of a hormone was probably first expressed by Mobius in 1906. He found that the blood of sheep neutralised the action of thyroid hormone.

Legiardi-Laura (1919-1923-1934) and Legiardi-Laura and Bruin (1929), treated horses with posterior pituitary preparations and claimed that the serum of such animals cured glycosuria and hypertension in many patients (Legiardi-Laura and Bruin, 1929).

The term "specific immune serum" was first used by Koyano (1923) to indicate antihormone substances produced experimentally in animals chronically treated with hormone extracts. He injected beef pituitary emulsions intraperitoneally into male rabbits and stated that the serum of such animals produced marked histological changes in the hypophysis of rats.

In 1924 Cotte published a series of experiments in which extracts of fowl ovaries were chronically injected into male rabbits. Thus a "serum anti-ovaire" was obtained which changed the colour of the plumage in hen-feathered cocks. Also in 1924 de Jongh observed an insulin inhibitory substance in the sera of chronically treated animals.

The name antihormone was used by Wiese (1928) who found that testicular extract coagulated the follicular fluid of nymphomanic cows but had no effect on the follicular fluid of normal animals. He attributed this to the formation of antihormones in the nymphomanic cow.

The most important demonstrations of the appearance of hormone antagonists in the blood as a result of pretreatment with a hormone preparation were published by Collip and Anderson (1934) for the thyrotropic, and by Selye et al (1934 a,b) for the gonadotrophic hormones.

A new phase of interest in the antihormone problem started with the discovery that upon continued administration to animals the action of certain hormones decreased considerably, in other words the animals became refractory.

This state of refractoriness was observed by Reiss and Reiss et al (1931), Zondek (1931), Collip (1932) and McPhail (1933).

In all of those experiments, heterologous pituitary preparations were injected in another species.

Theories of the antihormone factors.

The different concepts of the antihormone factors which apparently accumulated in the injected animals serum in response to the hormone administered, can be summarised as follows:-

1 - Abderhalden, the immunologist, (1918) described them as "Abwehr fermente" or protective enzymes appearing in the blood of experimental animals chronically treated with extracts of endocrine glands.

2 - Collip (1934) in his original statement linked the antihormone not to an antibody but to the "chalone" of Sharpey-Schafer.(1924). He suggested that in normal subjects there was an antagonistic principle for each hormone but it was not detected until it exceeded in amount the corresponding hormone.

3 - Simple exhaustion of the gonads or end-organs in general, or simple toxicity to these organs leading to loss of physiological response to continued hormone injections.

The fact that this phenomenon was actually due to a specific resistance to the hormone principles and not simply to an exhaustion of the end-organs was demonstrated by Selye et al,(1934b) and Okkels (1937).

4 - The immunological theory.

Experimental evidence produced so far, has led most of the authors to classify these inhibitory principles as immune bodies, which would make them comparable to antitoxins and anti-enzymes, all being antibodies that are

produced in animals in response to antigens.

The evidence for the antibody nature of antihormones is as follows:-

1 - The antihormones reside in the globulin fraction of the serum. (Thompson 1937, Harrington and Rowlands 1937, Hartman et al, 1940 a, b, Zondek & Sulman 1937).

2 - Gordon, Lowenstein and Charriper (1940) demonstrated that the site of production of antihormones is the same as that of other immune bodies, viz. the reticulo-endothelial system.

Collip and Anderson (1934) found that saline extracts of liver and spleen from refractory rats had antihormone actions.

3 - Okkels (1937) observed that the thyroid glands of animals refractory to the thyrotrophic hormone were not themselves refractory but can be restimulated. This stands against the idea of exhaustion or toxicity to the end-organ.

4 - In several respects they behave like antibodies:-

(a) They are not produced by homologous species extracts (Spence, Scourer and Rowlands 1938; Katzman, Wade and Doisy, 1947).

(b) They do not appear in parabiotic animals in spite of the prolonged hormone activity supplied by one animal to the other (Gordon et al.1940; Gordon 1941; Sulman 1937).

(c) Their rate of appearance and disappearance and accumulation in the blood in response to prolonged hormone injections, as well as their behaviour to a booster dose, fit them nicely among other immune bodies like anti-enzymes (Thompson, 1941).

Types of antibody reactions demonstrated with antihormone sera:

The presence of the antihormone factor was mainly demonstrable by its capacity to neutralise the hormonal activity in vivo (Selye, Bachman, Thomson and Collip 1934, Bachman, Collip and Selye 1934, Twombly 1936, Sulman 1937, Zondek and Sulman 1937 [illegible], Frend and Uyldert 1947).

In 1912, Rzent Kowski claimed to have obtained complement fixation reactions with the serum of hyperthyroid patients, using thyroid extract as an antigen.

In 1923, Hekton and his co-workers demonstrated that animals reacted to injections of thyroglobulin by producing species-specific precipitin immune bodies and in lesser quantities similar non-species-specific bodies.

A few years later, Hicks (1926) claimed to have produced antisera against "pure" thyroglobulin. He used the precipitin reactions of the antiserum to trace this substance in certain animal fluids. These claims were fortified by others with comparable results (Clutton et al 1937, 1938a, 1938b, Rotter 1936a, b; Picado & Rotter 1936, 1938, Went et al 1939, Anderson et al 1957, Werner et al 1961).

Precipitin reaction have also been demonstrated with antisera to the growth hormone (Hayashida and Li 1958), to insulin (Moloney et Aprile 1959, Wright 1959, 1960), to A.C.T.H.

(Cruickshank and Currie 1958) and to prolactin (Strangeways 1938).

New techniques for the qualitative analysis of precipitins have been developed (Ouchterlony 1946, 1949 1948, 1953, Oudin/1952), and have been used in investigating the specificity of antisera, cross reactions between different hormones, as well as the antigenic components, homogenecity and degree of purity of hormone preparations (Cruickshank & Currie 1958, Henry and VanDyke 1958; Rao & Shahani 1961, Butt et al 1961, Brody & Carlstrom 1960, 1961a,b).

Arquilla & Stavitsky(1956) showed that the serum of insulin treated rabbits and of some human diabetics would agglutinate sheep red cells coated with insulin. Since then agglutination of red cells coated with the corresponding hormone has been demonstrated with serum of rabbits treated with corticotrophin (Fishman et al 1959), somatotrophin (Read and Stone 1958), chorionic gonadotrophin(HCG)(Wide and Gemzell 1966) and thyrotrophin (Cline et al

1960). In each case agglutination is inhibited by prior addition of the homologous hormone to the serum and is not produced by normal serum.

This method was originally devised for the detection of insulin antibodies which do not usually precipitate the antigen.

In spite of the repeated reports about the immunological nature of the antihormones, several workers were not prepared to believe they were true immune bodies. Collip (1934, 1935 a,b, 1940) used the evidence given by Bachman et al (1934) that there was no correlation between precipitins and neutralising effects to argue against the antibody theory. Sulman (1937) showed that an antiserum against "pure" HCG did not give complement fixation reactions, while crude HCG did so with its own antiserum. Zondek, Sulman and Hockman (1938) observed that antihormone neutralised HCG quantitatively rather than by a process of adsorption, a quality which, they said distinguished this substance from known groups of immune bodies. However, they

concluded that antihormones were immune-bodies with unusual properties.

Although several workers failed to demonstrate antihormones in sera of animals chronically injected with homologous hormone extracts, others found them in comparable experiments. (Smith, 1930; Dushane et al 1935; Martins 1935; McCahey et al 1936; Twombly 1936; Collip 1937; Katzman 1937; Rowlands 1937; Thompson 1937; Anderson and Evans 1938).

The Specificity of Hormone Antibodies.

The attempt to correlate the classical antigen-antibody reactions (precipitins, complement fixation, etc.) given by hormone antisera with the neutralising power of these antisera led to conflicting results. Some workers found these were intimately related (Ehlich 1934; Twombly 1936; Fremery and Scheygrond 1937), while others found no relation at all between them, (Collip 1934, 1935 a,b, 1937; Bachman et al 1935; Thompson 1941;

Gegerson et al 1936; Van den Ende 1939).

Studying the specificity of the antihormones, including species specificity, hormone specificity and organ specificity, different workers produced opposite results. Some claimed they were species specific but not hormone specific (Gustus et al 1935; Picado and Rotter 1936, 1938; Cutting et al 1939; Meyer and Wolfe 1939; Van den Ende 1939). Others claimed they were hormone specific but not species specific (Meyer and Gustus 1935; Sulman 1937; Cope 1938; Yasuda 1938; Arquilla and Stavitsky 1956; Moloney and Goldsmith 1957; Wright 1959, 1960; Read and Bryan 1960), and others that they were even organ specific (Bachman 1934 a,b; Rowlands and Parkes 1934; Fluhman 1935 a,b).

At the other end of the scale, some authors claimed they were species specific and completely unrelated to the hormones themselves, but were produced against either contaminating antigens (Eichbaum et al 1935, 1937; Cutting et al 1939; Rowlands and Young 1939; Van den Ende 1939), or a protein carrier to which the hormone was

attached (Sulman 1937, Hartman 1940a, b; Cruickshank & Currie 1958).

This uncertainty of the nature of the antihormones and the failure to solve any of the main problems of the antihormone question are due to the unyielding controversy between the findings of different investigations.

A very serious handicap for earlier workers was the impurity of the hormone extracts then available to them. This problem was early realised by many workers (Ascheim and Zondek, 1928, Van den Ende 1939).

Claims using purified hormone extracts were made as early as 1935, when Meyer and Gustus produced neutralising antibodies in monkeys to purified thyroglobulin. In the past ten years chromatographic, electrophoresis and other techniques for the isolation of proteins and polypeptides have been introduced. Several experiments have now been carried out with "highly purified preparations", (Henry & Van Dyke 1958, Arquilla & Stavitsky 1956,

Fishman et al 1959, Levy and Samplimer 1961, and Cline et al 1960).

The hormones used have included three whose structures are known (corticotrophin, glucagon and insulin), two which have been purified but whose structures have not yet been defined (somatotrophin and mammotrophin) and others which have yet to be obtained in homogeneous forms (thyrotrophin and gonadotrophin) (Wright 1961).

The Antigonadotrophins.

Most of the early work on antihormones was done with gonadotrophic preparations.

It was early recognised that prolonged parentral administration of gonadotrophic hormone from human pregnancy urine gradually diminished the physiological effects of the hormone (Zondek 1931, Reiss 1931, Collip 1932, McPhail 1933, Evans 1939). This was believed to be due to the formation of an antigonadotrophic factor (Selye, Bachman, Thomson & Collip 1934, Bachman et al 1934).

The presence of this factor was demonstrable mainly by its capacity to neutralise the hormonal activity in vivo (Selye et al 1934, Bachman et al 1934, Twombly 1936, Sulman 1937, Zondek & Sulman 1937, Zondek & Sulman 1937, Gustus et al 1935, Gegerson 1930, Thompson & Cushing 1937, Brandt & Goldhamer 1936, Flulman 1935, Parkes & Rowlands 1936, Chow 1942, Frend & Uyldert 1947). The antihormone activity has been demonstrated by those workers with sera produced against human chorionic gonadotrophins, pregnant mare's serum, pituitary gonadotrophic hormone, interstitial cell stimulating hormone, prolactin, and human post-menopausal extracts.

Similarly precipitating and complement fixing antibodies have been obtained by immunising rabbits with gonadotrophic hormone preparations (Meyer et al 1935, Bachman 1935, Twombly 1936, Gegerson et al 1936, Picado 1936, 1939, Demancho 1937a,b, Eichbaumet al 1937, Thompson 1937, Van den Ende 1939, Got, Levy & Bouvillon 1959, McKean 1960, Brody and

Carlstrom1960, 1961a,b). Gel diffusion reactions of such antisera have also been demonstrated (Henry & Van Dyke 1958, Cruickshank & Currie 1958, Rao & Chahani, 1961, Butt et al 1961, Brody & Carlstrom1960, 1961a,b).

Wide and Gemzel (1960) obtained a haemoagglutination reaction with antigonadotrophic sera, and have used this technique for hormone assay as well as a pregnancy test.

It has long been appreciated that the purity of the gonadotrophic antigens play a decisive role in the attainment of the specificity of the antiserum. (Aschheim & Zondek 1928, Van den Ende 1939, Cruickshank & Currie 1958, Wright 1961).

In spite of several claims of successful purification of such preparations(Henry & Van Dyke 1958, McKean 1960, Butt and Round,1958;Butt et al. 1960,1961; Brody & Carlstrom1960, 1961àb)it is evident that attempts to prepare antigonadotrophic specific immune sera have been unsuccessful. Doubts of the chemical homogeneity of the

purified extract have been raised by Steelman et al (1957) and by Johnsen (1962).

Immunologically, similar doubts are based on the presence of cross reactions of the immune sera with other gonadotrophic preparations as well as with normal human sera and extracts from normal male urine (Bachman 1935, Henry & Van Dyke 1958, Brody & Witebsky, Rose & Shulman 1955, Brody & Carlstrom1961, Cruickshank & Currie 1958).

Doubts are also due to the results of absorption experiments (Van den Ende 1939, Cruickshank & Currie 1958) and to the failure to reduce the number of lines in gel diffusion (Henry & Van Dyke 1958, Brody & Carlstom 1961b, Butt et al 1961) by any method of purification.

Study of cross reactions between gonadotrophic hormone preparations from different sources has been contradictory (Butt et al 1961, Cruickshank & Currie 1958, Brody & Carlstrom1961).

It has also been suggested that a change

of the antigenicity of a hormone preparation either an increase or a decrease, will take place as a result of the process of purification (Wetner 1938a,b,c, Henry & Van Dyke 1958, Brody & Carlstom 1961).

It is therefore quite evident that little knowledge has been gained recently on the question of antihormones, especially where the gonadotrophins are concerned. This may be related to the purity of preparations, the chemical method employed and the nature of in-vivo tests.

The pursuit of the antihormone question is related to three main objectives -

a. The study of the nature of these immune bodies.

b. The possibility that some knowledge may be gained as to the nature of the hormone itself.

c. The application of antisera in an attempt to demonstrate sites of production and activity of the hormone.

It is with the purpose of throwing some

light on these problems that this work was started. Therefore the subject of study in this thesis in broad terms has been as follows:-

1. To study the immunological behaviour of a human urinary gonadotrophin preparation.
2. To study the nature and specificity of the antihormone reactions obtained, in order to show if they are one or different antibodies, to compare their specificities, their distribution in the plasma proteins, the difference between antisera produced against crude and against purified extracts.
3. To study the nature of the hormone antigen, how its antigenicity is affected by purification, whether serological methods are more hormone specific than chemical estimation of the glycoprotein hormone.
4. Lastly, an evaluation of the available serological techniques in the diagnosis, assay and tissue localisation of gonadotrophic hormones.

THE ANTIGENS.

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Introduction.

Many methods have been proposed for the preparation of gonadotrophins from human urine. The procedures differ considerably in nature and complexity. Usually however they suffer from the common disadvantages that they are laborious and tedious, that considerable loss of gonadotrophic activity may occur during their performance, and that the final extracts obtained are often toxic to the experimental animals (Loraine, 1957, 1958. Furthermore, the ability of different methods to recover the hormone activity from low-titre urines varies widely, and this decides their sensitivity.

Because of the growing interest in the antihormone problems, successful purification of gonadotrophic hormones has gained new importance.

The criteria of purity of the extracts have been considered as follows -

1. Bulk of the extract per litre or per 24 hour specimen of urine.

2. Solubility of the powder.

3. Toxicity of the extract to experimental animals.

4. Activity per mg. of the powder.

5. Homogeneity as shown by the behaviour of the extract in ultracentrifugation, electrophoresis and column chromatography.

In order to elucidate some of the antihormone problems, it was deemed necessary to study the difference in the antigenic behaviour between crude and purified extracts. The kaolin-acetone method of Loraine & Brown (1954) was chosen for the preparation of crude extracts, since it is a simple inexpensive procedure, less tedious and gives better total yields of gonadotrophins than the majority of published methods (Loraine & Brown, 1956a,b).

To obtain purer extracts, the tannic acid method of Johnsen (1958) was used. This is a modification of the original Levin

and Tyndale method (1936). It has also been shown to be simple and inexpensive, and to produce extracts containing little inert material. A 24-hour specimen yields a tannic acid extract weighing 20-30 mg.. as compared with 150-400 mg.. for the kaolin extract of such specimen. The percentage recovery of gonadotrophins from the urine is given for the kaolin KA method as 75% (Loraine 1957) and for the tannic acid method as 98% (Johnsen, 1958). The difference is most conspicuous in low titre urines.

Johnsen also demonstrated that his extracts were free of oestrogens and augmenting substances, unlike most of the extracts prepared by other methods (Johnsen, 1958). The toxicity of the extracts to mice is 10-20% for kaolin extracts and is five times less for the tannic acid extracts (Albert 1961). The mean activity per milligram of powder based on HMG20A Edinburgh preparation was found to be about 0.26 m.u.u. for the kaolin extracts,

and 6 m.u.u. for tannic acid preparation.

Other methods of purification were also used. Kaolin extracts were treated with tricalcium phosphate according to the method of Loraine & Brown (1954).

A kaolin preparation was fractionated with a saturated ammonium sulphate solution according to the method of Johnsen (1955). He was the first to try this technique on human urinary gonadotrophins, although Li et al (1949) and Van Dyke (1950) had used ammonium sulphate to purify extracts of swine pituitary glands.

Kaolin extracts and tannic acid extracts were further purified by gel filtration on columns of Sephadex G-25. This substance has been recently used to remove salts and low molecular weight material as one step in the purification of urinary and pituitary gonadotrophins (McShane & Meyer, 1961).

Biological Assay of Preparations.

Assays for gonadotrophic activity were performed by the infantile mouse uterus weight (m.u.u.) method of Klinefelter et al (1943), as modified by Loraine and Brown (1956). One mouse unit is defined as the activity causing a 100% increase of uterine weight in immature female mice (body weight 8-10 g.), injected intraperitoneally five times in the course of three days, with autopsy on the 5th day after the first injection. The total activity of the extract was calculated from the highest dilution (4 mice per dilution) producing such increase in the uterine weight.

H.M.G.-20A was not considered suitable for comparison with our extracts. Klinefelter and pregnancy urine extracts are of apparently different nature from postmenopausal extracts. The m.u.u. method is a very sensitive way of measuring the total activity of any of these extracts (Levin and Tyndale 1936). In this way it will be possible to correlate the biological activity of postmenopausal, pregnancy and pituitary gonadotrophins.

METHODS.

The Urinary Extracts:

Urinary Extracts prepared by the Kaolin Method:-

The Loraine and Brown kaolin-acetone method was used to prepare extracts of 24 hours urinary output from 85 separate sources. These sources were as follows:

1. 9 patients with the Klinefelter's syndrome. In 1942 Klinefelter, Reinfenstein and Albright described a syndrome which declared itself in adolescence, in the form of small testes, absent spermatogenesis, gynaecomastia, high urinary excretion of gonadotrophins and low excretion of 17-Ketosteroids. The 24 hour urinary output of gonadotrophins (mainly follicle-stimulating) in such cases was in the range of 40-160 m.u.u. as estimated by the method of Loraine & Brown (1956ab), (Stewart et al 1959).

Fresh interest was aroused in this syndrome, by the discovery that some but not all of these patients are chromatin positive (Plunkett and Barr, 1956).

2. 18 pregnant women mostly in their first

five months of pregnancy.

3. 15 normal males.

4. 26 normal females.

5. 12 post menopausal women.

6. 3 patients with signs of hypopituitarism.

7. Pooled urine, which showed proteinuria, from patients in the Sick Children's Hospital. Another pooled specimen was obtained from a children's nursery school and was free from chemically detectable protein.

In addition fourteen 24 hour urinary specimens were each divided into halves, one half was processed by kaolin extraction, plus treatment with tricalcium phosphate gel. The other half was processed by kaolin extraction, but the treatment with tricalcium phosphate was omitted.

The Kaolin-Acetone Method

The urine was centrifuged with Celite powder until it was clear. It was then adjusted to pH 4.0 with HCl. Kaolin powder (5 g. . for each litre of urine) was added.

The pH was rechecked, corrected if necessary, and the mixture stirred mechanically for one hour. After centrifugation the kaolin deposit was suspended in water and adjusted to pH 11-11.5 with NaOH. The suspension mixture was allowed to stand for 30 minutes with occasional shaking then centrifuged.

When it was planned to use tricalcium phosphate purification, the supernatant was adjusted to pH 8-8.5 and tricalcium phosphate suspension (3 ml. to each 100 ml.) was added. The mixture was mechanically stirred for 15 minutes, then centrifuged.

Whether tricalcium phosphate had been used or omitted the final supernatant was adjusted to pH 4-6 with HCl, and the gonadotrophins were precipitated with 5 volumes of acetone. The precipitate was collected, washed with absolute alcohol and ether, then dried in air.

The Tannic Acid Method

The urine sample was acidified roughly

to pH 4.0 by means of acetic acid. Sodium chloride was then added to the urine (10 g. for each litre) to increase the amount of flocculation. Tannic acid (20 ml. of 10% in water) together with Hyflo Super-Cel (10-12 g.) were added. After stirring mechanically, the suspension was poured into a Buchner funnel in order to produce a thick filter cake. This filter cake was used as a column in order to get a countercurrent set up for elution. Dilute tannic acid (0.1% solution) was passed through the cake, 96% alcohol was then passed through the cake in order to get rid of the excess of tannic acid and other materials. Following this a solution containing 10% ammonium acetate in ammonium hydroxide at pH 11.0 in 80% alcohol was applied. This changed the pH under cover of a high concentration of alcohol, keeping the active material insoluble and retained in the cake. This solution also removed the protein-bound tannic acid.

By lowering the percentage of alcohol from 80 to 40 in the same solution, the active constituents were eluted. The eluate was cooled and neutralised (approximately) by acetic acid. The concentration of alcohol was increased to 85% to precipitate the active material which was collected and washed with alcohol.

Results.

Table 1 gives a comparison of, in terms of activity, of preparations made by the kaolin and tannic acid methods. Thirteen urinary specimens from different sources were so tested. Each specimen was halved, one half being treated with kaolin, the other with tannic acid.

It will be seen that in every case the weights of the tannic acid extracts were approximately one-tenth that of the same urine processed by the kaolin acetone method. In six instances the total activity of the tannic acid extracts was less than that of the

corresponding kaolin extract. The remaining specimens gave identical results. Activity per unit weight however was always considerably higher in the tannic acid preparations.

Table I also provides the results of treatment of the kaolin extracts with tricalcium phosphate gel. These show that this process reduces the weight of the extract by 10 to 25 per cent with little or no reduction of activity. Obviously the material removed by the tricalcium phosphate technique is biologically inert.

In general it was found that extracts from any one patient were far from constant in activity. The output of gonadotrophin seemed to vary from time to time. One of the three hypo-pituitarism cases was a male patient who had been hypophysectomised because of carcinoma of the prostate. The urinary extracts from this case showed no biological activity. The other two cases were due to post-partum pituitary failure and the extracts assayed

between zero and 5 m.u.u. per 24 hours. No activity could be detected in the extracts of children's urine.

All these preparations were assayed in immature female mice according to the method of Loraine and Brown (1956).

Each extract was dissolved in 12.5 ml. of phosphate buffered saline (pH 7.2), and this constituted the neat concentration of the antigen. Doubling dilutions were made with the same solvent.

Salt Fractionation of Crude Extracts.

The saturated ammonium sulphate method for fractionating gonadotrophins from the urine, as described by Johnsen (1955), was used. Johnsen described two main fractions, one precipitated between zero and 55% saturation, and the other precipitated between 55 and 70 per cent saturation with ammonium sulphate.

A Kaolin extract of the urine of one of the Klinefelter patients (M) was fractionated with rising concentrations of saturated

Table I.

Comparison of Tannic Acid and the Influence of Tricalcium Phosphate treatment on Kaolin extracts.

Source.		Tannic Acid Purification.				Tricalcium PO_4 Treatment.			
		Kaolin Extract		Tannic Extract		Kaolin Extract		Tricalcium Phosphate treated extract.	
		Wt. extr.	M.u.u. 24 hrs.	Wt. extr.	M.u.u. 24 hrs.	Wt. extr.	M.u.u. 24 hrs.	Wt. extr.	M.u.u. 24 hrs.
Klinefelter	1	198	20	23	20	210	20	150	20
	2	225	80	24.6	40	230	40	205	40
	3	405	40	32	40	300	40	295	40
Pregnancy	1	275	80	18	80	395	80	312	40-80
	2	230	160	19.5	80	506	160	456	80
	3	360	80	26	40	280	80	210	80
	4	205	80	14.5	80	324	80	292	160 ?
	5	228	160	27	80	178	80	162	80
Normal male	1	120	10	12.7	5-10	209	10	194	5-10
	2	200	20	19	10	255	10	230	20
Normal female	1	180	10	20.5	10	172	10	170	10
	2	70	20	13	20	81	20	77	20
Menopausal	1	147.8	40	18.2	20				
	2					166	20	120	20-40
	3					105	40	101	40

Weight of extract in milligrammes.

ammonium sulphate solution (S.A.S.). Only half of the total powder (95 mg.. of 190 mg.) was thus treated. The other half was dissolved in 12.5 ml. of distilled water and used for comparison with the fractions in mouse uterus assays, precipitation reactions and gel diffusion.

Technique.

The Kaolin extract prepared for fractionation was stirred for one hour in 0.1 M KH_2PO_4 (pH 4.2-4.5). Undissolved residue (impurity) was redissolved in distilled water. To the supernatant, S.A.S. was added very slowly through a fine capillary attached to a burette. The mouth of the capillary was kept under the surface of the solution, and during the addition of S.A.S. the solution was vigorously stirred with a mechanical stirrer. In this way the proteins were prevented from coming into contact with the S.A.S. before it was thoroughly mixed up with the solution. The concentration of S.A.S. was slowly raised

stepwise. After each addition of S.A.S. the solution was allowed to stand at 3-4°C. for 24 hours to complete the precipitation. Even at high S.A.S. concentration the precipitate could be collected from the centrifuge. The precipitates were each redissolved in 12.5 ml. of distilled water.

Concentrations of S.A.S. are expressed as the percentage of S.A.S. present, and each fraction is designated with the saturation at which it was precipitated. A precipitate at half saturation is thus designated Fraction 50.

Results.

Table II shows clearly that all fractions had some biological activity, but the greatest activity resided in Fractions 55 and 70.

Purification of Hormone Extracts by Gel filtration on Sephadex Columns.

The following extracts were further purified on Sephadex after labelling them

Table II

Fraction.	M.u.u.
Original Kaolin Extract.	160
Undissolved residue. (Fraction I).	10
Fraction 55	40
Fraction 70	40
Fraction 100	20
Supernatant. (Fraction V).	Toxic

with fluorescein isothiocyanate:

a. A kaolin extract of urine from Klinefelter patient.

b. A kaolin extract of pregnancy urine.

c. A kaolin extract of postmenopausal urine.

d. A tannic acid extract of urine from a patient with the Klinefelter's syndrome.

e. A tannic acid extract of pregnancy urine.

f. Pregnyl (one ampoule containing approximately 80-160 m.u.u.).

g. Gestyl (one ampoule containing approximately 160-320 m.u.u.).

h. A tannic acid extract of children's urine was similarly treated.

Gel Filtration Technique.

A mixture of 2 ml. of the hormone solution and 2 ml. of a sodium carbonate-bicarbonate buffer (pH 8.5) was shaken for three minutes with 5-10 mg. of Celite containing 10% fluorescein isothiocyanate. The mixture was then centrifuged and the supernatant allowed to flow into a column of

of Sephadex G-25 measuring 2 x 20 cm. The column was then developed with phosphate buffer pH 6.5. Separation of the fluorescent fractions from the inert pale dye was observed almost immediately. The inert dye band remained very close to the point of application.

Results.

The following fractions were obtained on Sephadex gel. (Figure 1, Table III).

1. Five fractions were obtained from each of the Klinefelter extracts and the postmenopausal extract.
2. Four fractions were obtained from each of the pregnancy urine preparations (Pregnyl, tannic preparation and kaolin preparations).
3. Four fractions were also obtained from Gestyl (P.M.S.).
4. Three fractions were obtained from the children's urinary extract. The fractions were recognised by their colours.

In case of Klinefelter and post

menopausal extracts the first fraction (F.I) was pale orange, with a volume of 4-6 ml. The second fraction (F.II) was dark yellow, with a volume of 8-10 ml.

The third fraction (F.III) was dark orange with a volume of 8-10 ml.

The fourth fraction (F.IV) was similar to fraction two, but slightly less intensely yellow with a volume of 10-12 ml. The last fraction was very pale orange with a volume of 25 ml.

In the case of the pregnancy preparations, Fraction III with its intense orange colour was absent. The other fractions were obtained with the same volumes. The children's urinary extract showed fractions corresponding to F.I, F.II, and F.V of the Klinefelter's extracts.

Each fraction was tested for activity in mice. At least three batches of mice were used for each fraction, with two mice in each batch. Each mouse received 1/5th. of each

dilution, the activity being calculated according to the method of Loraine and Brown (1956).

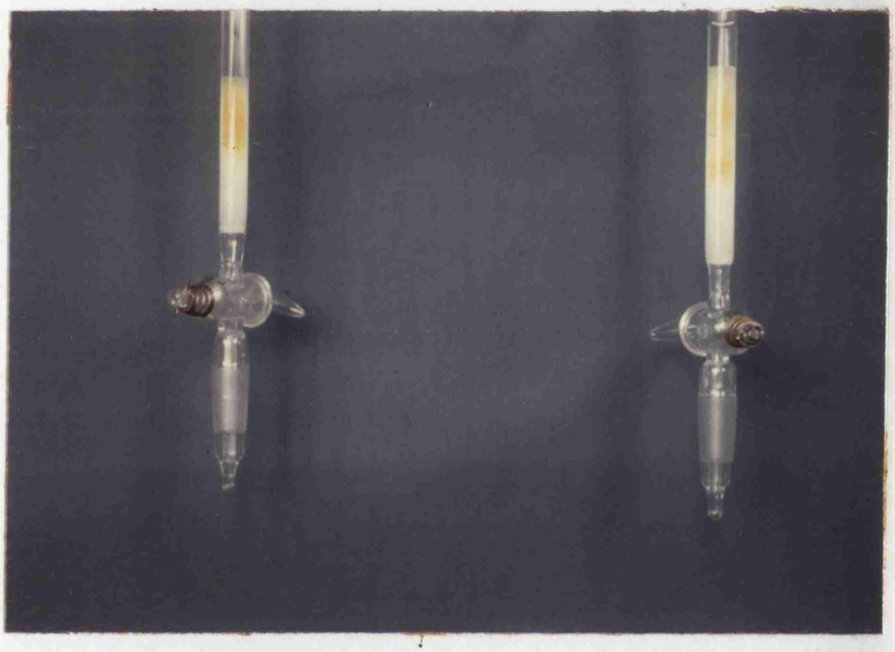
Table III shows that the biological activity was always shared between the first two fractions, except in the case of the post menopausal extract where only the first fraction showed activity.

However, the first fraction (F.I) was the more active one in the case of the Klinefelter extracts and the Gestyl preparation, while the second fraction was the more active one in the case of the pregnancy urine extracts including Pregnyl.

Although apparently similar fractions were obtained from children's urine, these were biologically inactive, indicating that separation on Sephadex is related to a group of substances rather than to gonadotrophins per se.

Table III

Preparation	Method of Extraction.	Total muu.	Fraction.	muu.
Klinefelter.	Kaolin	20	1	10
			2	5
			3	-
			4	-
			5	-
Klinefelter.	Tannic ā	20	1	5-10
			2	5
			3	
			4	
			5	
Pregnancy.	Kaolin	20	1	5
			2	10
			3	-
			4	-
			5	Absent
Pregnancy.	Tannic ā		1	5
			2	10
			3	-
			4	-
			5	Absent
Menopausal.	Kaolin	10	1	10
			2	-
			3	-
			4	-
			5	-
Gestyl. (Pregnant mare's serum)	Organon Ltd.	80-160	1	80
			2	20
			3	-
			4	-
			5	Absent
Pregnyl. (Pregnancy urine).	Organon Ltd.	40-80	1	20
			2	40
			3	-
			4	-
			5	Absent



I II

Figure I. Sephadex Fractionation.

I. Gel filtration of fluorescein labelled pregnancy urine extract, showing four fractions. Top fractions = pale orange Second and Third = deep yellow Fourth = pale orange.

II. Gel filtration of a fluorescein labelled extract of KF urine, showing five fractions. Top fraction = pale orange Second = deep yellow, then Third = deep orange (appearing pale in the photo because of the blue background). Fourth = deep yellow. Fifth = pale orange.

The Influence of pH on Kaolin extraction.

The following variations of the standard kaolin procedure were adopted in order to determine whether a) the chemical process altered the antigenic properties of the extract, and b) the subsequent immunological reactions bore any relationship to biological activity.

Two specimens from two Klinefelter patients were halved. One half of each specimen was adsorbed on kaolin at pH 4.0 and eluted at pH 11-11.5 according to the standard method. The other half was adsorbed at pH 5.0 and eluted at pH 10.0. According to Loraine & Brown (1954) this latter treatment leads to loss of most of the biological activity.

Similarly one menopausal and one pregnancy urine were halved. One half of each was adsorbed and eluted by the standard technique. The other half was adsorbed at pH 8 and eluted at pH 11-11.5.

The effect on biological activity of these procedures is shown in Table IV. Immunological results are detailed in a later part of the thesis.

Table IV.

Specimen.	Extraction Method.	Biological Activity. (m.u.u.)
Klinefelter 1	Standard	40
	Adsorbed pH 5 Eluted pH 10	5
Klinefelter 2	Standard	80
	Adsorbed pH 5 Eluted pH 10	5
Menopausal	Standard	40-80
	Adsorbed pH 8 Eluted pH 11	Nil
Pregnancy	Standard	320
	Adsorbed pH 8 Eluted pH 11	Nil

Summary:

1. Kaolin extracts of urine contained more biologically inert material than tannic acid preparations. However, the total activity of the purer extracts was sometimes less than that of the crude ones.

2. Purification of kaolin extracts with tricalcium phosphate eliminated only some biologically inactive material and slightly affected the biological activity.

3. Purification of kaolin extracts by salt fractionation produced several fractions each of them possessing some biological activity. The most active fractions were precipitated at salt saturation of 55% and 70%.

4. Purification of kaolin and tannic acid extracts by gel filtration on Sephadex G25 produced the same fractions for each hormone regardless of the original method of extraction. Only the first two fractions of each extract had biological activity in mice. The most active fraction was the second fraction in case of

pregnancy urine extracts, and the first fraction in case of non-pregnancy extracts.

Conclusions:

Chemical purification and gel filtration of hormone extracts eliminate some inert ingredients, but also cause some loss of total activity of an extract, although the activity per milligram powder rises.

Salt fractionation of hormone extracts showed that fractions to be discarded as impurities actually possess some biological activity. The activity seems to be distributed among several components of the extract.

IMMUNOLOGICAL EXPERIMENTS.

IMMUNOLOGICAL EXPERIMENTS

Preparation of antisera.

Young adult female cross-bred rabbits kept on a diet of rabbit pellets supplemented with fresh greens twice a week were used for the production of antisera. Each rabbit was kept in a separate cage.

Injection schemes:

1. Our main procedure was to immunise the rabbits frequently to obtain high titre antisera within a short time, hoping they would be more specific.

A volume of 2 ml. of $\frac{1}{4}$ dilution of the antigen preparation (which in the case of biologically active preparations contained 1.5-2 m.u.u.) was injected subcutaneously twice daily for one week, twice weekly for the succeeding two weeks, then once weekly thereafter. In the case of Pregnyl and Gestyl, each dose consisted of half an ampoule. Whenever an animal was about to be bled an intravenous injection of the

antigen was given in the ear vein and bleeding was carried out at least two hours later.

The details of the animals treated are as follows:-

3 rabbits were immunised with Kaolin-acetone (KA) extracts of urine from Klinefelter patients. (KFK).

3 rabbits with tannic acid extracts of similar urines. (KFT).

2 rabbits with KA preparations of pregnancy urine. (CGK).

2 rabbits with tannic acid preparations of pregnancy urine (CGT).

2 rabbits with Pregnyl, 1/2 ampoule (750 I.U.) per dose.

2 rabbits with Gestyl, 1/2 ampoule (500 I.U.) per dose.

In addition to these highly active preparations, four less active extracts were used -

1 rabbit was treated with Kaolin extract of

normal male urine.

1 rabbit was treated with tannic acid extract of normal male urine.

1 rabbit was treated with Kaolin extract of the urine of children under the age of five. (Ch.K).

1 rabbit was treated with tannic acid extracts of children's urine. (Ch.T).

2. Ramon's Adjuvant was tried in three rabbits. All three had produced low titre antisera with the above method of immunisation. They were then immunised with the adjuvant to compare the effectiveness of the two methods. One rabbit received 3 ampoules of Pregnyl (4500 I.U.) in 2 mls. of adjuvant twice weekly. One rabbit received three ampoules of Gestyl (3000 I.U.) in 2 mls. of adjuvant twice weekly. The third rabbit received 5 m.u.u. of KFT extracts in 2 mls. adjuvant twice weekly.

Bleeding.

The bleeding was done by penetrating

the marginal ear vein with a Hagedorn needle after shaving both surfaces of the ear. The rabbit was immobilised in a specially designed cage. Ten ml. were the maximum quantity obtained from each animal at any bleeding. The bleeding was done once every week at first then as required.

The blood was received in universal containers which had been sterilised by autoclaving, and dried in the hot air oven to remove all moisture. The blood was incubated for thirty minutes at 37°C then left overnight at 4°C. Next morning the serum was centrifuged off and pipetted in 1-2 ml. aliquots in small test tubes. The antiserum was either used at once if required, or else a little merthiolate was added to the serum which was then kept in the deep freeze until needed, (minus 40°C).

SEROLOGICAL TESTS

Precipitin Reactions:

Introduction:

Since the precipitin reactions are of great classical importance in immunology, in addition to the simplicity of the techniques involved, it is natural that they should be tried in the antihormone study. In order to achieve reproducible results and to use such reactions for the titration of varying amounts of hormone antigens and antibody, it is necessary to carry out preliminary experiments. Such experiments will indicate the optimal conditions for the hormone - antihormone systems. When using rabbit sera for precipitation reactions in the zone of antibody excess or in the equivalence zone (zone of optimal antigen-antibody ratio) no antigen is detectable in the supernatant, and the precipitate contains all the antigen. (Heidelberger & Kendall, 1929). When more antigen is added to the optimal ratio

reaction, more precipitation occurs, usually because antigen-antibody complexes already formed join each other. Therefore, maximal precipitation occurs usually in the zone of antigen excess up to a certain point. This maximal precipitation in the zone of antigen excess has been found a most useful measure (as useful as the optimal ratio point) of the concentration of this antigen in many precipitating systems (Wilson and Miles, 1957, Libby 1938a, b, 1947, Pope & Healy, 1938, Bolton et al, 1948). The phenomenon has been recently used for quantitations of beta-lipoproteins in human sera by means of the immunocrit method (Heskell et al 1961, Chaney et al 1961).

No antigen or antibody can be detected in the supernatant when precipitation is complete at the zone of equivalence in most systems. Occasionally, however, complete absorption in the equivalence zone leaves some antibody in the supernatant, and a second

addition of antigen may be required to precipitate the remaining antibody (Wilson & Miles, 1957). Apart from these general provisos, it has been found that complete precipitation in the gonadotrophin-antigonadotrophin system, especially outside the zone of equivalence, requires standing several hours at room temperature (Cruickshank & Currie, 1958).

Preliminary Experiments:

I. Detection of the Antigen-Antibody Optimal Ratio:

The interfacial-ring technique was used. Varying dilutions of the antigen were layered gently on top of undiluted antiserum. The volumes of the reagents were measured and added by the dropping technique, one drop being one volume. Altogether, ten sealed pasteur pipettes were set for each test as follows:

Tube No.	1	2	3	4	5	6	7	8	9	10
Antigen (neat volume).	3	2	1	$\frac{1}{2}$	$\frac{1}{4}$	$\frac{1}{8}$	1/16	1/32	1/64	1/128
Antibody (neat volume)	1	1	1	1	1	1	1	1	1	1

The first tube to show a ring of precipitation at the interface indicated the optimal antigen concentration.

Results:

Most of the antigens used showed an optimal reaction at a dilution of 1/3 to 1/4, with antisera obtained after two to three weeks of immunisation. Some pregnancy urine extracts gave an optimal reaction at a dilution of $\frac{1}{2}$.

One extract (child II) gave optimal reactions when undiluted.

Titration of the Antisera.

The same technique as used for the detection of optimal ratios was used for the titration of different antisera, but in this case, the antigen concentration was kept constant (chosen as the optimal antigen concentration for the particular system) and the antiserum concentration was varied in the following manner:-

Tube No.	1	2	3	4	5	6	7	8	9	10
Anti-serum (one volume)	Neat	½	¼	⅛	1/10	1/20	1/50	1/100	1/250	1/500
Antigen (one volume)	¼	¼	¼	¼	¼	¼	¼	¼	¼	¼

The reaction was read after allowing to stand at room temperature.

Titration of the antisera was done for the following purposes -

1. To show the effect of varying the time allowed for the reaction on the final titre of the reaction.
2. To follow the rise and fall of precipitating antibodies in the circulation of rabbits after variable periods of immunisation.
3. To study the effect on the titre of using an adjuvant in the immunisation of rabbits.

1 - The Effect of Time on the Reaction:

In order to find out the optimal time for the reaction between gonadotrophins and antigonadotrophic sera, in precipitation reactions, a series of tests were performed

using the interfacial ring technique. The reaction was read after ½ hour, 2 hours and overnight standing at room temperature.

The optimal time of reaction was tested for antigen-antibody mixtures, in which a constant optimal antigen dilution was added by the interfacial ring technique, to an equal volume of varying dilutions of antiserum. The result was indicated by the highest serum dilution showing the reaction.

Results.

Although the mixture containing optimal antigen and antibody concentrations always gave a quicker reaction (within half an hour) yet when the antigen was in excess a reaction appeared at higher titre of antiserum when the result was read after two hours and overnight standing.

2. The Rise and Fall of Precipitin Titre.

The precipitin titre of antiserum to extracts from urines of Klinefelter syndrome

patients (KFT) was tested in the interfacial ring technique, at intervals. The titres of reactions of this antiserum were tested against crude and purified extracts prepared from urine of Klinefelter syndrome patients. These reactions were performed at one, three, five and ten weeks from starting the immunisation, and at similar intervals after stopping the injections. Similarly, antisera to pregnancy extracts, normal male extracts, children's extracts, Pregnyl and Gestyl were titrated.

Results.

Using a tannic acid preparation of gonadotrophins (nominally "pure") for injections, a perceptible precipitin reaction was obtained after one week. The titre was estimated at 1 in 4 using a kaolin extract of a Klinefelter urine as the antigen. A tannic acid extract of the same urine gave a titre of 1 in 2.

At the end of 3 weeks of injections

both antigens gave a titre of 1 in 100 with the rabbit serum. Further increases were noted at 5 weeks. In the case of the Kaolin antigen the titre was 1 in 500, but with the tannic acid antigen it was 1 in 250. Further immunisation did not appear to increase the titre, and indeed after 10 weeks of injections the concentration of antibodies tended to diminish.

Cessation of injections caused a rapid fall in antibody concentration and after 3 weeks the titre was only 1 in 10. No reaction at all could be detected by the interfacial ring technique in another 2-3 weeks using the kaolin extract as the reacting antigen. The purer tannic acid antigen still gave a reaction at that time but this too disappeared rapidly thereafter.

The sera from three rabbits immunised against extracts from three Klinefelter patients showed the same pattern. The results for one such preparation are shown in Table 5.

Table V.

Rise and Fall of Precipitin Titre.

Antiserum to	Antigen used for titration.	Rise of Titre.				Cessation of Immunisation.	Fall of Titre.			
KFT	KFK	4	100	500	500		100	10	0	0
	KFT	2	100	250	250		100	10	0	0
CGT	CGK	8	500	500	1000		250	50	0	0
	CGT	2	1000	250	500		100	50	0	0
Normal male T	Normal male K	4	20	50	100		20	0	0	0
	Normal male T	2	10	50	100		10	0	0	0
Child T	Child K	2	10	20	20		4	N	-	-
	Child T	0	4	8	8		2	0	-	-
Gestyl	Gestyl	0	8	8	20		2	0	0	0
Interval in weeks.		1	3	5	10		1	3	5	10

KFT = Tannic acid extract of Klinefelter urine.
CGT = Tannic acid extract of pregnancy urine.
Normal male T = Tannic acid extract of male urine.
KFK = Kaolin extract of Klinefelter urine.
CGK = Kaolin extract of pregnancy urine.
Normal male K = Kaolin extract of male urine.
Child T = Tannic acid extract of child's urine.

When the antiserum to normal male extracts was tested against its antigen, a progressively rising titre was obtained with both Kaolin and tannic acid extracts. The titre obtained with the KA antigen was reasonably high from the start, reaching a maximum of 1 in 100 after ten weeks of continuous immunisation, and disappearing completely after three weeks of stopping the injections. However, the titre obtained with tannic acid antigens, started at a rather low level of 1 in 2, rose considerably after 5 weeks, and reached 1 in 100 after 10 weeks. No reaction could be obtained with this antigen 3 weeks after the immunisation was stopped.

When antiserum to children's extracts reacted with KA children's extracts, the titre was 1 in 2 after one week, and rose to 1 in 20 after 5 weeks of continuous immunisation. A tannic acid children's extract, gave no

precipitation until the third week, when the titre was 1 in 4. The titre reached 1 in 8 after 5 weeks. The rate of fall of the titre of this antiserum was studied, until the third week, when no reaction could be detected.

When the titre of antiserum to pregnancy urine extracts was tested with KA extracts of such urine, it was found to be 1 in 8 after one week, 1 in 500 after 3 weeks, and between 1 in 500 and 1 in 1000 after 10 weeks of immunisation. Tannic acid extracts of pregnancy urine gave a titre of 1 in 2 after 1 week, 1 in 250 after 3 weeks, and 1 in 500 after 10 weeks of continuous injection. When the immunisation was stopped, there was a quick drop of the titre of the reaction reaching 1 in 50 for both KA and tannic preparations after 3 weeks. No reaction could be detected with either preparation after two further weeks.

Anti Gestyl serum produced a precipitin

reaction with Gestyl only after three weeks of immunisation. The titre then was 1 in 8 and rose to 1 in 20 after 10 weeks. Once the injections were stopped the titre dropped to 1 in 2 after one week, and no reaction was obtained after three weeks.

3 - Effect of adjuvant on the precipitin titre:

Reference to Table VI shows the effect on the anti-hormone titre of changing over to Ramon's adjuvant in the immunisation of three rabbits. After three weeks of immunisation without adjuvant, anti-Pregnyl serum reacted with Pregnyl up to a titre of 1 in 4, anti-Gestyl with Gestyl up to 1 in 4 and anti-KFT with KFT up to 1 in 8. When immunisation had been carried on for another three weeks, using mixtures of adjuvant and hormones, the titres became, Neat, 1 in 4, and Neat respectively.

Although the response of the rabbit receiving KFT was probably poor from the start (producing a titre only 1 in 8 after three weeks) yet the response of the other two

Table VI

Effect of adjuvant on precipitin titre.

Animal	Antigen Injected.	Titre after 3 weeks without adjuvant.	Titre after 3 weeks with adjuvant.
1	Pregnyl.	1 in 4	Neat
2	Gestyl.	1 in 4	1 in 4
3	Klinefelter (K).	1 in 8	Neat

rabbits was low as expected when using highly purified antigens (Pregnyl and Gestyl).

However, there was a drop in the precipitin reactions of all antisera after adjuvant immunisation of the rabbit.

Cross Precipitation Experiments:

Titration of the precipitin reactions of different antisera was performed for the following purposes:

1. To demonstrate qualitatively and quantitatively cross reactions of the different antisera and the preparations tested.
2. To study the species specificity of the precipitin reaction by testing Gestyl (pregnant mare's serum) and its antiserum against human extracts and their antisera.
3. To study the hormone specificity of the reaction by clarifying the following points:

a. The relation between the titre of the reaction and the hormone activity of the antigens.

b. The titre of reaction of antisera to

biologically inert extracts when tested against inert and active extracts.

c. The effect of purification of the antigens on the titre of its reaction with different sera.

Results.

As can be seen from Table VII Gestyl did not give any cross precipitation with other antisera, nor did its antiserum precipitate other antigens. This indicates that the precipitin reaction of antihormone sera is species specific.

All the other antisera tested gave positive precipitin tests with all the antigens of human origin, whether the source was the placenta (HCG), or the pituitary gland (KF, normal male, HMG).

Preparations which were completely inactive in mice produced considerable precipitin reactions with all the antisera except the anti-Gestyl serum. These inactive extracts included children's extracts, extracts obtained

Table VII.

Cross Precipitation Titres.

Antiserum to	m.u.u.	Klinefelter Tannic	Pregnancy Tannic	Gestyl.	Child Tannic.
Antigen					
Klinefelter (K)	80	560	500	-	50
Klinefelter (T)	80	250	100	-	10
Pregnancy (K)	160	500	500	-	25
Pregnancy (T)	160	100	250	-	10
Menopausal (K)	80	250	100	-	25
Menopausal (T)	40-80	100	250	-	10
Male (K)	20	100	100	-	10
Male (T)	10-20	25	10	-	10
Child (K)	0	16	8	-	10
Child (T)	0	2	Neat	-	4
Gestyl	640	-	-	8	-
Pregnyl	320	4	4	-	Neat

at unusual pH from pregnancy urine (CG 8), from menopausal urine (MG 8) and one post menopausal extract rendered inactive by storage for a long time. From Table VII it can be clearly seen that antiserum to children's extracts gave strong reactions with active and inert extracts. Although the titre of reaction of highly active extracts (KF, CG, MMG) was often higher than the titre of reaction of less active extracts, yet the opposite sometimes occurred. The discrepancy between the titre of reaction and biological activity was most marked with antigens of no activity (extract 342 and children's extract), and antigens of low activity (normal male and normal female extracts). At the same time purified extracts usually gave less precipitation than their crude counterparts, whether reacting with their own antiserum or other antisera. This was equally true of biologically active and biologically inert extracts.

Conclusions.

The precipitin reactions of antihormone sera seem to be species-specific but not organ-specific. They also seem to be at least partly hormone-specific since (1) anti-hormone sera always gave stronger reactions than antiserum to children's extract, (2) the biologically active extracts often reacted more strongly than biologically inert ones, and (3) purification of the hormone extracts did not abolish any of these reactions. However, considerable reactions were obtained with inactive extracts and their antisera.

Microprecipitation (Immunocrit) Experiments:

Since estimation of the titre of cross precipitin reactions proved disappointing for our purpose, an attempt was made to estimate these reactions by measuring the amount of precipitate by the immunocrit method.

The technique used was that employed for the serological quantitation of serum lipoproteins (Heskell et al 1961, Chaney et

al 1961, Bergquist et al 1961). On a clean dry glass plate, one drop of antiserum and two drops of antigen (¼ or ½ according to the optimal concentration) were mixed together thoroughly with a glass rod for about 10 seconds. The mixture was then allowed to flow up the capillary tube to the two inch mark. The tip of the tube further away from the fluid level was sealed by rotating it slowly in the flame of a bunsen burner. Then the tube was centrifuged. The amount of precipitate was read with the aid of a magnifying glass and a special millimeter scale. Each 0.25 mm. of precipitate was reported as one plus.

Preliminary Experiment:

To compare the amount of precipitate with the titre of reaction as indicators of the extent of the precipitin reaction, a combined immunocrit and interfacial ring experiment was conducted. The reactions were read after ½ hour, 2 hours, and over night standing at room

temperature.

As can be seen from Table VIII, after a reaction time of ½ hour, the interfacial ring technique showed a positive reaction with some extracts, while the immunocrit technique did not show any reaction. Thus the interfacial ring technique is apparently more sensitive. However, reading the reaction after two hours showed complete agreement between the interfacial ring and the immunocrit techniques. Allowing the reaction to proceed overnight, the changes in the titre of reaction and the amount of precipitate were again equal in extent and pattern. Overnight at room temperature was adopted as the method of choice. A titre of ½ - 1/10 corresponded with +, a titre of 1/20 - 1/50 corresponded with ++ to +++ and a titre of 1/100 - 1/250 corresponded with +++ to ++++.

Cross Precipitation Reactions by the Immunocrit Method:

Analysis of the results in Tables VIII and

Table VIII.

Comparison of titre and amount of precipitate.

Effect of time of reaction on both. (using pure antiserum and antigens).

Preparation.	M.u.u.	Amount of precipitate with variation of time.			Titre with variation of time.		
		½ hr.	2 hrs.	over-night.	½ hr.	2 hrs.	over-night.
CG 1	160	±	+	++	N	1/20	1/50
CG II	320	±	+	++	N	N	1/10
CG III	320	++	+++	++++	1/20	1/100	1/250
CG IV	160	+	++	+++	1/2	1/10	1/10
CG V	320	-	++	+++	1/20	1/50	1/50
CG VI	160	+	+++	+++	1/10	1/50	1/50
CG VII	160	-	+	+	-	1/2	1/5
CG VIII	over 160	+	++	+++	1/5	1/20	1/20
CG 8	0	++	++	++	1/50	1/50	1/50
KFT	10	+	++	++	1/10	1/10	1/20
KFK 1	20-40	+	++	+++	1/5	1/20	1/50
KFK II	20	++	+++	+++	1/5	1/20	1/50
KFK III	80	++	++	+++	1/50	1/250	1/250
Normal male 1	20	-	++	+++	1/2	1/5	1/10
Normal male II	20-40	+	++	++	1/2	1/5	1/20
Normal male III	10-20	-	+	++	-	1/2	1/2
Normal female T	10	-	++	++	1/2	1/5	1/10
Normal female T		-	+	+	N	1/2	1/2
HMG	80-160	-	±	++	N	N	1/5
HMG 8	0	+	+	++	1/5	1/10	1/10
Child 1	0	+	++	++	1/5	1/20	1/20
Child II	0	-	+	+	N	1/2	1/4
342	0	++	+++	+++	1/50	1/50	1/100
Pregnyl	160	-	±	+	N	N	1/5
Pregnyl	160	-	-	+	-	N	1/2

CG = Pregnancy urine.
CG 8 = Pregnancy urine extracted at pH 8.
KFT = Tannic acid extract of Klinefelter urine.
KFK = Kaolin extract of Klinefelter urine.
HMG = Human Menopausal Extract.
HMG 8 = Menopausal urine extracted at pH 8.
342 = Extract from hypophysectomised male.

Table IX.

Cross Precipitin Reactions (Immunocrit)

Source.	M.U.U.	KFS	KFTS	CGS	CGTS	PrS	NoS	ChS	GesS
Kaolin Extracts.									
CG I	160	++++	++	++	+	+	++	+	-
CG II	320	+++	+	++	+	++	+	+	-
CG III	320	+++	++	++	++	+	++	++	-
CG IV	160	++	+	+	+	+	+	+	-
CG V	320	++	+	+	+	++	+	+	-
CG VI	160	+++	++	+++	+	++	++	+	-
CG VII	80	++	+	++	+	+	+	++	-
CG VIII	160	++	+	+	-	-	+	-	-
CG pH 8	0	+++	++	++	+	+	+	+	-
Pregnyl	320								-
KFK 1	80	+++	++	+++	++	++	+	++	
KFK 2	160	++	+	+	+	-	+	+	
KFK 3	40	+++	++	++	++	++	++	+	
KFK 4	80	++	+	+	+	+	+	+	
KFK 5	80	+++	++	++	+	+	++	++	
KFK pH 5	0-5	+++	++	++	+	+	+	++	
KFK pH 5	5	++	+	+	+	+	+	+	
N♂ I	20	+	+	+	+	-	+	+	-
N♂ II	20	++	+	++	+	-	++	+	-
N♂ III	10	++	+	++	+	-	+	+	-
N♂ IV	20	++++	+++	+++	+	+	+	+	-
N♂ V	20	+++	++	++	++	+	++	+	-
342	0	+++	++	+++	0	+	++	?	-
N♀ I	20	+	+	+	+	+	-	+	-
N♀ II	10	++	+	+	+	+	+	+	-
N♀ III	40	++	++	++	+	+	+	+	-
N♀ IV	10-20	++	+	+	+	-	+	-	-
N♀ V	20	+	-	+	+	-	+	+	-
HMG	80	++	+	+	-	-	+	-	
HMG 8	0	++	++	++	+	-	+	-	
Child I	0	++	+	++	+	+	++	+	
Child II	0	+	+	+	+	+	+	+	
Gestyl	∈40								

KFS = Antiserum to Kaolin extract of Klinefelter urine.
KFTS = " " Tannic " " " "
CGS = " " Kaolin " " Pregnancy "
CGTS = " " Tannic " " " "
PrS = Antiserum to Pregnyl.
NoS = Antiserum to Male urine extract.
ChS = Antiserum to child extract.
GesS = Antiserum to Gestyl.

342 = Extract from hypophysectomised male.

Table IX (contd.)

Source.	M.U.U.	KFS	KFTS	CGS	CGTS	PrS	NoS	ChS	GesS
Tannic Extracts.									
CG I	160	+++	++	+++	+	+	+	++	-
CG II	320	+++	++	++	+	+	++	++	-
CG III	320	++	+	++	+	+	++	+	-
CG IV	160	++	++	+	+	-	+	+	-
CG V	320	+	+	++	++	++	+	+	-
CG VI	160	++	++	++	++	++	++	+	-
CG VII	80	++	+	+	+	+	+	+	-
CG VIII	160	+	+	+	+	-	+	+	-
CG pH 8	0								
Pregnyl	320	++	++	++	++	+	+	-	-
KFK 1	80	+++	++	++	++	+	+	+	
KFK 2	160	++	++	++	+	+	+	-	
KFK 3	40	++	+	+	+	-	+	-	
KFK 4	80	++	+	-	-	-	+	-	
KFK 5	80	++	+	++	-	-	+	-	
KFK pH 5	0-5								
KFK pH 5	5								
No♂ I	20	+	+	+	-	-	+	-	
No♂ II	20	+	+	+	+	-	+	+	
No♂ III	10	++	++	++	+	-	++	+	
No♂ IV	20	++	+++	++	++	+	+	-	
No♂ V	20	+++	+++	++	+	+	++	-	
342	0								
No♀ I	20	+	+	+	+	+	-	-	
No♀ II	10	+	+	++	+	-	+	+	
No♀ III	40	++	+	+	-	-	-	-	
No♀ IV	10-20	++	++	++	+	+	+	+	
No♀ V	20	+	+	+	+	+	+	+	
HMG	80	+	+	+	-	-	+	-	
HMG 8	0								
Child I	0	++	+	+	+	-	+	++	
Child II	0	+	+	-	-	-	+	+	
Gestyl	640	-	-	-	-	-	-	-	+

CG = Pregnancy urine.
CG pH 8 = Pregnancy urine extracted at pH 8.
KFK = Kaolin extract of Klinefelter urine.
KFK pH 5 = Klinefelter urine extracted at pH 5.
HMG = Human Menopausal Extract.
HMG 8 = Menopausal urine extracted at pH 8.

normal male, normal female, inactive menopausal, inactive pregnancy extracts) seemed to give too big a precipitate in many cases, whether purified or crude, and whether reacting with crude or purer antisera.

Fractions separated by the Sephadex gel method were tested against antisera to Klinefelter tannic extract and pregnancy tannic extract. The results are shown in Table X.

There was a rough correlation between biological activity and the amount of immunocrit precipitate but the reactions were obviously not specific and cross reactions were obtained in every case apart from Gestyl which gave no reactions with either antiserum.

Similar tests were carried out on the fractions obtained by salting out a tannic acid preparation of Klinefelter urine with saturated ammonium sulphate solution. In this case the preparations were tested against the corresponding antiserum. The immunocrit readings and precipitin titres of the various

Table X.

Immunocrit Precipitin reactions of Sephadex Fractions.

Preparation.		Antiserum to:- Klinefelter Tannic.	Pregnancy Tannic.
Klinefelter Kaolin (20 muu)	1	++	+
	2	±	-
	3	-	-
	4	-	-
	5	-	-
Klinefelter Tannic (20 muu)	1	+	+
	2	±	-
	3	-	-
	4	-	-
	5	-	-
Pregnancy Kaolin (20 muu)	1	+	+
	2	-	-
	3	-	-
	4	-	-
Pregnancy Tannic (20 muu)	1	+	+
	2	-	-
	3	-	-
	4	-	-
Menopausal Kaolin (10 muu)	1	+	+
	2	±	-
	3	-	-
	4	-	-
	5	-	-
Gestyl (160 muu)	1	-	-
	2	-	-
	3	-	-
	4	-	-
Pregnyl (80 muu)	1	++	++
	2	-	-
	3	-	-
	4	-	-

fractions are given in Table XI.

Again there is a rough correlation between biological activity and the precipitin tests but in view of the scatter of activity over the fractions no specificity can be attributed to these reactions.

Conclusions:

The immunocrit technique has no advantage over the interfacial titration technique except its simplicity. Both are equally reliable when the longer reaction times are used. The general pattern of the results of cross precipitation tests was the same in both methods.

Conclusions on Precipitin Reactions of Anti-Hormone Sera.

The precipitin reactions of the anti-hormone sera tested, proved to be species-specific, but not organ-specific. They also seemed to be only partly hormone specific, since biologically inert component or components of the antigenic extracts appeared

Table XI.

Fractions separated by ammonium sulphate tested against corresponding antiserum.

Antigen	Biological Activity	Precipitin titre	Immunocrit Reading.
	m.u.u.		
Klinefelter (T)	160	250	++++
Insoluble Fraction	10	100	++
Fraction 55	40	250	+++
Fraction 70	40	250	+++
Fraction 100	20	50	+
Supernatant	Toxic	0	-

to be equally involved in the reactions. Therefore, the degree of reaction could not serve as an accurate indication of the biological activity of an extract, whether the reaction was measured by the titre or by the amount of precipitate. Antisera to inert extracts, also reacted with active and inert extracts, more so with the former.

Purification of the extracts used for the production of antisera and/or for cross reactions, diminished but did not abolish the so-called non-specific reactions.

Complement Fixation Experiments.

Introduction.

The complement fixation reactions are like the precipitin reactions one of the most classic techniques in immunological work. It has been shown to be more sensitive than precipitation (Boyd, 1961). Since the precipitin tests tended to give us false impressions about the gonadotrophin-antigonadotrophin system, it seemed wise to try complement fixation reactions in this system.

The technical application of this reaction demands a very accurate standardisation of each reagent. Before using both antigen and antiserum the anticomplementary action of each of them was determined. From this the appropriate dilution for both to be used in the test was determined.

In our tests we used the constant volume technique of Wasserman. Equal volumes of antigen, antibody and complement solutions were added to each other, the variant being either

the amount of complement, or the dilution of antiserum contained in this standard volume. As in Wasserman's original technique the standard volume was 0.1 ml.

Preliminary Experiments:

1. Titration of the Antisera.

Doubling dilutions of antiserum were tested against a constant dilution of the antigen which had been used to produce the antiserum. The serum dilutions used were as follows - 1/4, 1/8, 1/16, 1/32, 1/64, 1/128, 1/256, 1/512, 1/1024, 1/2048.

No antiserum was anticomplementary at 1/4 dilution, so this dilution was chosen as the starting dilution. The antigen dilution was chosen as the lowest dilution not possessing anticomplementary power. This was usually 1/4 or 1/8.

2. The rise and fall of complement fixation titre.

All the antisera tested for precipitin reaction were also tested for their complement

fixation reactions. Reference to Table XII shows that the titre of complement fixation rose with the period of immunisation. Antiserum to tannic acid extracts of Klinefelter urine gave a titre of 1/2 after 1 week, and 1/32 after three weeks, when tested against the corresponding antigen. These titres are lower than the corresponding titres of precipitation for the same system. However, by the end of the 5th. week of immunisation, the complement fixation titre exceeded the precipitin titre, reaching a maximum of 1/2048. After 5 more weeks, there was a slight drop in both complement fixation and precipitin titres. After stopping the immunisation the complement fixation titre dropped quickly, but a positive reaction at 1 in 2 serum dilution was still obtained after 10 weeks, unlike the findings for precipitation tests.

When a kaolin extract was used for the titration of antiserum to tannic extracts, the pattern was rather different. Higher

Table XII.

Rise and Fall of Titre of Complement Fixation.

Antiserum to	Antigen used for titration.	Rise of Titre.				Cessation of Immunisation.	Fall of Titre.			
KFT	KFK	2	64	2048	2048		512	16	2	0
	KFT	2	32	2048	1024		512	64	8	2
CGT	CGK	8	1024	2048	4096		2048	512	16	4
	CGT	2	512	2048	4096		1024	512	32	8
Normal male T	Normal male K	2	64	256	256		16	2	0	0
	Normal male T	N	8	64	128		8	2	0	0
Child T	Child K	2	16	64	64		4	0	0	0
	Child T	0	8	64	32		N	0	-	-
Gestyl	Gestyl	N	4	16	64		8	N	-	-
Interval in weeks.		1	3	5	10		1	3	5	10

KFT = Tannic acid extract of Klinefelter urine.
CGT = Tannic acid extract of pregnancy urine.
KFK = Kaolin extract of Klinefelter urine.
CGK = Kaolin extract of pregnancy urine.
CGT = Tannic acid extract of pregnancy urine.
Normal male T = Tannic acid extract of male urine.
Normal male K = Kaolin extract of male urine.

titres were obtained until the end of the 10th. week of immunisation, while a quicker drop occurred after cessation of immunisation. Table XII also shows that antiserum to tannic acid extracts of pregnancy urine when tested against the corresponding antigen had titres of 1/2, 1/512, 1/2048 and 1/4096 after periods of immunisation of 1, 3, 5, 10 weeks respectively, which were again higher than the corresponding precipitin titres. After stopping the injections the titre dropped until a positive reaction was obtained only at 1 in 8 dilution after ten weeks. When a kaolin extract was used for the titration of the same antiserum, a stronger reaction was obtained up to the 5th. week of immunisation. Afterwards the titres for both kaolin and tannic preparations were similar.

Antiserum to Gestyl reached a maximum titre of 1 in 64 after 10 weeks of immunisation and dropped to zero after 5 weeks of stopping the injections. Antiserum to normal male

extracts tested against its corresponding antigen reached a maximum titre of 1/256 after five weeks of continuous injection. The same titre was obtained after five more weeks. When injections were stopped the titre became 1 in 2 after three weeks and no reaction was obtained after five weeks. Antiserum to children's extract reached a maximum titre of 1 in 64 after five weeks of immunisation and no reaction was obtained three weeks after the immunisation was stopped.

In general the purer normal male and children extracts reacted with their antisera to a lower titre than cruder extracts.

3. The Effect of Adjuvant on the complement fixation titre.

Reference to Table XIII shows that higher titres of complement fixation of anti-Pregnyl and anti-KFT sera were obtained before and after the rabbits received an adjuvant with the immunising antigens. However the titre of the anti-Gestyl serum was unaffected,

Table XIII.

Effect of Adjuvant on Complement Fixation Titres.

Antigen Preparation.	3 week titre without Adjuvant.	3 week titre with Adjuvant.
Pregnyl.	16	4
Gestyl.	64	64
Klinefelter (K)	32	4

as in the case of the precipitin titre of the same antiserum.

Cross Complement Fixation Reactions.

The Antiserum Titration Technique:

Analysis of the results in Table XIV indicates that cross reactions occurred between all antisera and all antigens used including Gestyl and anti-Gestyl serum. Therefore, the complement fixation reaction of the hormone-antihormone system seems to be neither species specific nor organ specific.

The hormone specificity of these reactions seems doubtful. Although biologically active extracts usually gave strong reactions, yet biologically inert or low activity extracts (children's, normal male, normal female, inactive pregnancy, inactive menopausal, 342 (hypophysectomised male) extracts) produced considerable reactions with all antisera. Also, kaolin extracts generally produced stronger reactions than the purer tannic acid preparations. Although, in general

Table XIV

Cross reaction titres in Complement Fixation Tests using Antisera to Tannic Acid Extracts.

Antigen.	m.u.u.	Antisera to Klinefelter Tannic	Pregnancy Tannic	Gestyl Tannic	Child Tannic.
Klinefelter (K)	80	1024	512	64	32
Klinefelter (T)	80	512	512	32	16
Pregnancy (K)	160	512	1024	16	16
Pregnancy (T)	160	256	512	32	4
Menopausal (K)	80	512	128	16	4
Menopausal (T)	40-80	512	64	8	16
Male (K)	20	256	16	4	16
Male (T)	10-20	128	32	4	8
Child (K)	0	32	16	0	64
Child (T)	0	16	4	0	16
Gestyl.	640	32	16	16	0
Pregnyl.	320	8	16	8	4

each antigen reacted with its own antiserum to a greater extent than other antigens did, yet antiserum to children's extracts gave stronger reactions with the more biologically active preparations.

It is clear that the titre of complement fixation can be correlated with hormone activity of the antigens within a range of error similar to that of the precipitin titre. The error is most marked when testing low activity or inert extracts.

Tables VII and XIV show that although the complement fixation titres in general were higher than the corresponding precipitin titres, in case of anti-child-extract serum, precipitin reactions were of higher titres than complement fixation reactions.

Complement Variation Technique.

In attempt to overcome the discrepancies obtained with the titration technique, we tried Harrison's quantitative technique as modified by Wyler. A standard volume of 0.1 ml. of each

reagent was used. Constant dilutions of antigen and antiserum were used. These dilutions were the lowest dilutions not showing spontaneous anti-complementary activity. The amount of complement was varied by varying the number of minimal haemolytic doses (M.H.D.), contained in the standard volume. Two M.H.D. were used for the antigen and antiserum control tubes. Ten tubes were used for the test proper containing the following numbers of M.H.D. of complement: $2\frac{1}{2}$, 3, 4, 5, 6, 7, 8, 9, 10, and 11. The result was reported as the serial number of the last tube showing a positive reaction (partial lysis or no lysis).

Results.

These are shown in Tables XV and XVI. The first tables lists the reactions obtained using crude Kaolin extracts of urines as the antigens against antisera to both crude and purified extracts. In this case there was little evidence of specificity. Extracts from five Klinefelter patients gave strong

reactions with anti-Klinefelter serum but strong reactions were also obtained with anti-pregnancy serum. Reactions with "Gestyl" which, biologically, has some of the characters of pituitary gonadotrophins, were, on the other hand, rather weak. Similar anomalies were apparent in all other groups. In addition it is quite evident that there was no relationship between the biological activity of the extract and the consumption of complement even when tested against specific antisera.

"Pure" extracts (Table XVI) gave reactions which showed promise of a little more specificity but it was of trifling nature and again there was only very rough correlation with biological activity.

Sephadex Preparations.

The fractions obtained by this method were tested against two antisera according to the complement consumption technique. The antisera were those prepared against Klinefelter and pregnancy tannic extracts. Two Klinefelter

Table XV.

Complement Consumption tests using crude extracts against antisera to crude and purified antigens.

Antigens. Kaolin Extracts.		Antisera to M.U.U.	KK	KT	PK	PT	MK	CK	Pregnyl	Gestyl
Klinefelter	1	40-80	5	3	3	2	3	1	2	2
	2	160	7	3	5	3	3	3	2	5
	3	40	6	3	6	4	3	2	3	4
	4	40-80	9	9	3	4	5	3	2	2
	5	80	8	6	8	5	0	2	5	3
Pregnancy	1	160	3	3	4	3	3	0	3	3
	2	320	7	2	5	3	5	2	2	1
	3	320	5	3	8	5	6	4	4	2
	4	160	5	4	5	1	2	2	4	3
	5	320	3	2	5	3	4	1	3	2
	6	160	3	2	2	2	1	0	2	4
	7	80-160	10	7	9	6	2	0	7	5
	8	320	8	5	9	5	2	2	2	6
pH	8	0	6	2	6	2	1	0	1	1
Menopausal	1	80	5	4	5	2	3	3	2	5
	2	40	10	7	10	4	3	1	5	3
	3	80	3	5	2	3	3	1	2	4
	4	160	1	3	3	2	3	0	1	4
	5	160	10	4	8	2	1	0	4	2
	6	40-80	3	3	1	0	1	2	2	0
pH	8	0	4	4	5	2	1	2	2	1
Male	1	20	5	3	3	0	4	0	2	2
	2	20-40	6	2	5	3	4	2	1	1
	3	10-20	5	3	3	1	2	3	1	0
	4	20	5	4	3	2	2	1	0	2
	342	0	5	5	6	3	2	3	1	2
Female	1	10-20	3	2	1	2	3	2	2	0
	2	10	6	5	5	0	1	0	1	1
	3	40	2	2	3	2	0	2	0	0
Child	1	0	7	5	4	2	3	3	2	1
	2	0	2	2	1	0	2	2	0	1

KK = Klinefelter Kaolin.
KT = Klinefelter Tannic.
PK = Pregnancy Kaolin.
PT = Pregnancy Tannic.
MK = Male Kaolin.
CK = Child Kaolin.

The result is expressed as the serial number of the last tube showing a positive reaction.

Table XVI.

Complement consumption tests using purified extracts against antisera to crude and purified antigens.

Antigens: Tannic acid extracts.		M.u.u.	Antisera to: KK	KT	PK	PT	MK	CK	Pregnyl	Gestyl
Klinefelter	1	40-80	5	4	2	3	2	2	3	4
	2	160	8	6	8	3	2	2	5	5
	3	40	3	3	4	2	3	2	0	1
	4	40-80	7	5	4	5	2	1	2	3
	5	80	8	5	5	3	5	2	3	2
Pregnancy	1	160	3	3	4	3	3	1	4	2
	2	320	5	2	3	3	2	1	2	2
	3	320	8	7	10	6	3	0	6	4
	4	160	2	1	6	6	1	0	8	3
	5	320	3	3	2	1	2	2	3	2
	6	160	4	2	3	3	1	1	3	2
	7	80-160	5	5	8	6	2	1	6	3
Menopausal	1	80	6	4	3	2	2	3	2	5
	2	40	6	5	3	4	5	3	4	1
	3	40-80	3	2	3	2	1	2	2	1
Male	1	20	3	2	2	1	3	2	1	2
	2	20-40	4	2	3	0	2	3	2	0
	3	10-20	3	3	2	0	2	0	0	1
	4	20	5	5	3	2	1	1	2	2
Female	1	10-20	3	2	2	1	2	1	1	1
	2	10	3	2	0	2	2	0	2	0
	3	40	2	2	0	0	0	2	0	0
Child	1	0	5	5	3	2	3	3	0	2
	2	0	2	3	2	0	1	2	1	2
Gestyl		640	2	2	2	1	2	0	4	5
Pregnyl		320	2	2	3	2	2	0	4	3

KK = Klinefelter Kaolin.
KT = Klinefelter Tannic.
PK = Pregnancy Kaolin.
PT = Pregnancy Tannic.
MK = Male Kaolin.
CK = Child Kaolin.

The result is expressed as the serial number of the last tube showing a positive reaction.

menopausal, gestyl and pregnyl were tested in this manner. The results are shown in Table XVII.

The reactions were always, with one exception, most marked with the first fraction. This is in contrast to the biological activity which almost always was strongest in the second fraction. Again there was little evidence of specificity.

Conclusions on the Complement Fixation Reactions.

Although the complement fixation reactions of the Gonadotrophin-antigonadotrophin system, proved to be more sensitive than the corresponding precipitin reactions, yet they did not appear to be more hormone-specific. Like the precipitin reactions they are not organ-specific, but unlike the latter they also are not species specific. The rate of appearance and disappearance of the two reactions, as well as the comparison of the titres, seem to suggest that the two reactions may not necessarily involve the same antibody or the same factors.

Table XVII.

Antigen. (Sephadex Fractions).		M.u.u.per Fraction.	Antisera to:- Klinefelter Tannic	Pregnancy Tannic
Klinefelter (Kaolin)	1	10	6	4
	2	5	2	0
	3	0	3	0
	4	0	0	0
	5	0	0	0
Klinefelter (Tannic)	1	10	4	2
	2	5	3	0
	3	0	3	1
	4	0	0	0
	5	0	0	0
Pregnancy (Kaolin)	1	5	6	6
	2	10	4	3
	3	0	1	2
	4	0	0	0
Pregnancy (Tannic)	1	5	2	4
	2	10	4	5
	3	0	2	3
	4	0	0	0
Menopausal	1	10	8	3
	2	0	2	2
	3	0	0	0
	4	0	0	0
	5	0	0	0
Gestyl	1	80	2	3
	2	20	2	2
	3	0	0	1
	4	0	0	0
Pregnyl	1	20	2	1
	2	40	0	0
	3	0	0	0
	4	0	0	0

Haemoagglutination Experiments.

Introduction:

The disappointing lack of specificity of the two classical reactions, precipitation and complement fixation in immunological studies has been attributed, at least in part, to the relative insensitivity of those reactions (Wright 1961). As a result other techniques, apparently more sensitive and more specific have recently been introduced. Of these the haemoagglutination technique of Roitt (1958) and Roitt et al (1956) has been applied to the HCG-anti-HCG system by Wide and Gemzel (1961) with favourable results. We conducted a study of this technique on all our antisera and antigens.

Method.

Different antigens were each adsorbed on Group O, Rhesus Negative human red cells with the aid of tannic acid. Of a 1% suspension of these antigen-sensitised RBCs., a volume of 0.1 ml. was added to 0.4 ml. of rising dilutions

of different antisera. The pattern of the RBC sediment, after overnight standing at 4°C. indicated the result of the test.

The antigens were used in a constant dilution of 1/4. Antisera were first tested for their spontaneous ability of causing non-specific agglutination of tanned, but not sensitised RBCs. It was found that a dilution of 1/10 of any antiserum used did not cause such non-specific reactions. Therefore, when titrating antisera, the following serum dilutions were used - 1/10, 1/20, 1/50, 1/100, 1/250, 1/500, 1/1000, 1/2500, 1/5000 and 1/10,000. The results were expressed as the reverse of the highest dilution giving a positive agglutination of RBCs sensitised against a given antigen. Thus a titre of 1/100 was expressed as 100. For each test a serum control tube was set by adding 0.2 of antiserum 1/50 to 0.2 of the corresponding antigen (undiluted) then 0.1 of the antigen sensitised RBCs. No agglutination should occur in this

tube if there was no non-specific reactions.

Preliminary Experiments.

1. The Rise and Fall of Haemoagglutination Titre:

Reference to Table XVIII shows that the titre of the haemoagglutination reaction of an antiserum to tannic acid preparation of KF urine rose steadily with the period of immunisation to a maximum of 1/10,000 after 5 weeks, using a crude antigen for the titration. When using a purified extract, the maximum titre of reaction was 1/5000. The haemoagglutination titre was higher than the precipitation and complement fixation titres throughout.

After stopping the injections, the titre seemed to drop slowly. After 10 weeks the reaction was positive at 1/10 dilution of the antiserum. During this fall, there was no difference in titre of reaction when the antiserum reacted against crude or purified extracts. This fall was much slower than that observed in precipitin and complement fixation reactions.

Table XVIII.

Rise and Fall of Haemoagglutination Titre.

Antiserum to	Antigen used for titration.	Rise of titre.				Cessation of Immunisation.	Fall of titre.			
KFT	KFK	1000	5000	10.000	10.000		5000	1000	100	10
	KFT	250	2500	5000	5000		5000	1000	50	10
CGT	CGK	250	5000	10.000	20.000		5000	2500	2500	50
	CGT	250	5000	10.000	10.000		5000	1000	1000	20
Normal male T	Normal male K	10	100	500	1000		50	20	0	0
	Normal male T	10	50	500	1000		50	10	0	0
Child T	Child K	0	20	100	250		50	0	0	0
	Child T	0	20	50	250		10	0	0	0
Gestyl	Gestyl	10	100	500	1000		50	10	0	0
Interval in Weeks.		1	3	5	10		1	3	5	10

KFK = Kaolin extract of Klinefelter urine.
KFT = Tannic acid extract of Klinefelter urine.
CGK = Kaolin extract of pregnancy urine.
CGT = Tannic acid extract of pregnancy urine.
Normal male T = Tannic acid extract of male urine.
Normal male K = Kaolin extract of male urine.
Child T = Tannic acid extract of child's urine.
Child K = Kaolin extract of child's urine.

Antiserum to tannic acid preparations of pregnancy urine (CGT), gave titres of 1/250, 1/5000-1/10,000, and 1/10,000 after 1, 3, 5 weeks respectively, whether the titration was done with tannic or kaolin pregnancy extracts. The titre after 10 weeks often rose to 1/20,000, an extra dilution of the antiserum being added to the test. After stopping the injections, the titre dropped to 1/1000 after 5 weeks and 1/20 at the end of ten weeks. During the fall of titre, especially after the third week, the tannic acid preparations sometimes gave lower titre than kaolin extracts. Antiserum to normal male extracts produced a titre of 1/1000 after ten weeks of immunisation which dropped quickly until no reaction was detected 5 weeks after the injections had been stopped. Antiserum to children's extracts showed a maximum titre of 1/250 after 10 weeks of immunisation but showed no reaction three weeks after the injections had been discontinued. With most of these antisera kaolin extracts

seemed to give higher titres than those given by tannic extracts, especially in the later stages of immunisation and during the period of fall of titre.

Anti-Gestyl serum had a slowly rising titre which reached a maximum of 1/1000 after ten weeks of continuous injections and which dropped to 1 in 10 after three weeks of stopping the injections, disappearing completely after two more weeks.

These results seem to indicate that the haemoagglutination reactions are not only more sensitive than precipitation and complement fixation but also somewhat more hormone specific. Antisera to biologically inert extracts show a very low titre compared to antisera to active extracts. Also the level of the titre of the latter type of antisera appears to be in proportion to the activity of the antigens. The rate of disappearance of the reactions also seems to be inversely proportional to the antigen activity. However, we still found sometimes

that cruder extracts gave stronger haemoagglutination reactions than purer ones when both were tested against the same antiserum.

2. Effect of Adjuvant on Haemoagglutination Titre.

From Table XIX it can be seen that while the haemoagglutination titre of anti-KFT sera dropped, the titre of anti-Gestyl and anti-Pregnyl sera continued to rise after Ramon's adjuvant had been added to the immunising material. It will also be noticed that under the same circumstances, the anti-Gestyl precipitin titre dropped while its complement fixation titre was unaffected (Tables VI and XIII). This may suggest that the three reactions do not necessarily involve one and the same antibody. This is also suggested by the drop in the precipitin and complement fixation titres of the anti-Pregnyl serum while its haemoagglutination titre continued to rise. (Tables VI, XIII and XIX).

Table XIX.

Effect of adjuvant on Titre of Haemagglutinins.

Hormone injected.	3 week titre without Adjuvant.	3 week titre with Adjuvant.
Pregnyl.	50	100
Gestyl.	500	1000
Klinefelter (Kaolin).	250	10

Cross Haemoagglutination Experiments.

1. The Haemoagglutination Titration Technique.

The results recorded in Table XX show that cross reactions occurred between all antigens and all antisera used including Gestyl (P.M.S.) and anti-Gestyl serum. Anti-child-extract serum also reacted with all the antigens, while children's extracts reacted with all antisera.

The titre seemed to be higher where each antigen reacted with its own antiserum, than when other antigens cross reacted with this antiserum.

The haemoagglutination reaction is much more sensitive than the complement fixation and precipitation reactions. Also the titre of haemoagglutination agrees with the biological activity of the extract more closely than the titres of the other reactions. Using purified extracts instead of crude extracts reduced the titre of reaction in most cases, with the exception of pregnancy extracts. In the latter instance

Table XX.

Cross reactions in Haemagglutination Tests.

Antigen.	muu.	Antisera Klinefelter Tannic.	Pregnancy Tannic.	"Gestyl".	Child Tannic.
Kaolin Extracts.					
Klinefelter.	80	5000	1000	100	10
Pregnancy.	160	2500	5000	250	20
Menopausal.	80	5000	1000	50	10
Male.	20	100	50	50	50
Child.	0	50	20	10	20
Tannic Extracts.					
Klinefelter.	80	2500	1000	100	10
Pregnancy.	160	2500	5000	50	20
Menopausal.	40-80	2500	500	10	50
Male.	10-20	100	50	50	10
Child.	0	10	10	50	20
Proprietary.					
Gestyl.	640	250	250	50	0
Pregnyl.	320	500	500	10	0

the titre of reaction was the same for both crude and purified extracts when reacting with antisera to Klinefelter and pregnancy extracts. The main disagreement between the haemoagglutination titre and the m.u.u. activity of the antigens occurred with extracts of low biological activity. Even with highly purified proprietary preparations such as "Gestyl" and "Pregnyl" cross reactions occurred. It is notable that the titres obtained with anti-Klinefelter and anti-pregnancy sera were the same for "Gestyl" and a similar results was obtained with Pregnyl. At the same time the titres obtained with Pregnyl were double those shown by Gestyl although the biological activity per mg. of the latter was twice that of the former. This could mean that there is a common antigen in these two substances and that the amount is greater in Pregnyl. A similar relationship might also be indicated in the Klinefelter and pregnancy extracts.

One curious result is the low titre given

by Gestyl with its own antiserum. A possible explanation is that there are impurities in Gestyl which have little antigenic power hence the low titre with the corresponding antiserum. These impurities however, may be very similar although not identical to those contained in our own tannic and Kaolin extracts, hence the high titres with anti-Klinefelter and anti-pregnancy sera. The lack of antigenic power of these impurities may be related to the chemical process involved in manufacture of these preparations.

The low titres shown by all preparations when reacting with anti-Gestyl serum might be thought at first to be related to hormone activity but they do not correspond to the biological activity. Inactive preparations gave the same titres as highly active extracts. Therefore although the haemoagglutination proved to be an improvement over precipitation and complement fixation by being more sensitive and apparently more hormone

specific yet it could not abolish cross reactions between hormone extracts and antisera to inert extracts, or between anti-hormone sera and biologically inert extracts. Nevertheless a study of the cross reactions of the extracts of high potency suggested that the antigens of "pituitary" origin e.g. Klinefelter and menopausal, differed in character from those of placental type, and in the latter case this technique might be used as a practical test provided suitable dilutions of the antiserum were made. In order to overcome "non-specific reactions" a further modification of the haemoagglutination technique was tried.

2. Cross Reactions by the Haemoagglutination Inhibition Technique.

The Boyden (1951) technique as modified by Wide and Gemzel (1960) was used. Equal volumes of antigen and antiserum were mixed together in test tubes (0.2 ml. of each). To each tube was then added 0.1 ml. of a 1% suspension of antigen-sensitised red blood cells. In our experiments, a constant

serum dilution of 1 in 10 was used in all tubes. The dilutions of the antigens were varied as follows: 1/5, 1/10, 1/20, 1/40, 1/80, 1/160, 1/320, 1/640, 1/1280, 1/2560, ten tubes being used for each antigen. Occasionally, one or two extra dilutions were required (1/5120, and 1/10,240). For each test a control tube was set in which was added 0.2 ml. of the antiserum (1/10) to 0.2 of normal Saline, and 0.1 ml. of the suspension of the RBCs sensitised to the corresponding antigen. A haemoagglutination reaction occurring in this tube rules out the possibility of non-specific inhibition in the test proper. The results were expressed in terms of the dilution factors, taking the end point as the last tube showing no haemoagglutination.

Results.

From Tables XXI and XXII it is apparent that there are cross reactions between all antigens and all antisera. There is little

Table XXI

Haemagglutinin Inhibition Test.

Kaolin Antigens.		M.u.u.	Antisera to - KK	KT	PK	PT	MK	CK	Pregnyl	Gestyl
Pregnancy	1	160	2560	640	2560	80	10	40	160	20
	2	320	2560	320	2560	640	10	10	40	10
	3	320	320	80	160	40	20	0	20	40
	4	160	640	80	320	320	5	10	80	80
	5	320	1280	80	80	80	20	5	10	80
	6	160	1280	160	640	160	20	40	80	20
Klinefelter	1	40	40	20	80	20	5	0	0	40
	2	80	320	320	640	20	10	10	40	20
	3	40-80	640	40	160	80	10	80	40	10
	4	40-80	40	10	10	10	20	0	10	10
	5	160	2560	160	160	20	10	20	5	80
	6	0	80	10	20	5	10	20	0	10
	7	0	160	20	20	10	40	10	5	5
Male	1	20	640	160	640	20	10	10	20	0
	2	20-40	80	160	80	20	80	20	0	10
	3	10-20	40	40	20	40	20	20	10	0
Female	1	10-20	160	20	40	10	20	5	20	0
	2	10	40	10	20	5	10	5	5	0
Menopausal	1	80	160	10	40	10	10	80	20	40
	2	40	2560	1280	320	320	20	20	20	80
	3	40-80	80	10	20	10	20	20	10	0
	4	0	20	5	20	10	40	10	20	5
Child	1	0	80	20	20	5	10	5	5	0
	2	0	10	10	0	0	10	0	0	5

KK - Klinefelter Kaolin.
KT - Klinefelter Tannic.
PK - Pregnancy Kaolin.
PT - Pregnancy Tannic.
MK - Male Kaolin.
CK - Child Kaolin.

Table XXII

Haemagglutinin Inhibition Test.

Tannic Antigens.		Antisera to - H.u.u.	KK	KT	PK	PT	MK	CK	Pregnyl	Gestyl
Pregnancy	1	160	320	80	320	80	10	20	40	20
	2	320	1280	640	2560	640	20	10	10	10
	3	160	640	20	80	80	10	10	20	5
	4	320	320	80	160	40	10	10	40	80
	5	160	40	20	160	40	20	20	40	0
	6	160	640	160	2560	80	20	10	80	10
Klinefelter	1	40	40	40	10	20	5	0	10	10
	2	80	80	80	640	80	0	20	40	10
	3	40-80	160	80	160	40	10	10	40	20
	4	40-80	40	20	10	0	10	10	10	20
	5	160	160	160	320	20	10	5	5	20
	6	0	5							
	7	0	20							
Male	1	20	10	20	10	10	20	20	10	0
	2	20-40	20	10	20	10	20	10	10	10
	3	10-20	40	20	20	10	5	10	10	0
Female	1	10-20	10	10	5	5	10	0	0	0
	2	10	10	10	10	5	10	0	0	0
Menopausal	1	80	80	40	40	20	40	10	20	40
	2	40	160	160	160	80	20	5	20	10
	3	40-80	40							
	4	0	0							
Child	1	0	40	10	20	5	5	5	0	0
	2	0	10	0	5	0	0	0	0	0
Pregnyl		320	20	10	40	10	10	10	20	80
Gestyl		640	160	20	80	40	20	10	80	2560

KK - Klinefelter Kaolin.
KT - Klinefelter Tannic.
PK - Pregnancy Kaolin.
PT - Pregnancy Tannic.
MK - Male Kaolin.
CK - Child Kaolin.

evidence of an organ and specific reaction. Some antigens gave higher titres with antisera other than their own. For example, in Table XXI it can be seen that the third, fifth and sixth Kaolin pregnancy antigens gave higher titres with anti-Klinefelter sera than they did with anti-pregnancy sera. Similar cross-reactions were shown by other antigens. Purification of the antigens by the tannic acid method (Table XXII) produced a reduction in the titre of all reactions but did not appear to improve the specificity of the reaction. It did, however, reduce the titre of extracts of low or no biological activity. There was some correlation between titre and biological activity in that there was a clear differentiation between highly active extracts and those with little activity. Comparison of the various extracts of high activity however, showed that this correlation was of a rough nature. It is clear that the antigens involved are multiple and the reaction is not

entirely related to hormone activity. A practical point does emerge however. In normal female patients it would be possible to use a test of this nature for the diagnosis of pregnancy. The dilution factor of antigens, particularly in relation to tannic acid antigens, is sufficiently high compared to non-pregnant antigens, to warrant the use of the test in this way. The titre of normal female tannic antigens against antisera to Kaolin or tannic extracts of pregnancy urine varied from 1 in 5 to 1 in 10. The lowest titres of pregnancy antigens with the same antisera were 1 in 80 and 1 in 40 respectively. Similarly a rough segregation could be carried out of Klinefelter from normal males.

Little can be deduced as to the nature of the antigens. It would seem from the results obtained with highly purified hormone preparations like "Gestyl" that part of the antibody is due to actual hormone, but it is obvious that other antigens are involved.

This is also apparent from experiments with fractions obtained from Sephadex columns. It will be remembered that five fractions were obtained from most preparations. Biological activity was usually confined to the first two fractions eluted, and fraction 1 was the more active. In the case of menopausal extracts only the first fraction was active and in pregnancy extracts the second fraction was more active than the first.

Table XXIII shows that the haemoagglutination inhibition titre is higher with the active fractions. This is most apparent with tannic acid antigens. There was however, considerable reaction with inactive fractions indicating the presence of antigens other than hormones. Table XXIII also shows that the first fraction of Gestyl (PMS), reacted more strongly with anti-Klinefelter serum than with anti-HCG serum. The first fraction of Pregnyl did the opposite. This may indicate that Gestyl is immunologically closer to Klinefelter extract than HCG extracts. Moreover, although fraction II of Pregnyl was the more active biologically, yet it produced less haemoagglutination inhibition than fraction I. This suggests that Haemoagglutination inhibition is not absolutely dependent on the biological activity.

Table XXIII

Haemagglutinin inhibition titres of Sephadex Fractions.

		Klinefelter Tannic	Pregnancy Tannic
Klinefelter (Kaolin)	1	80	80
	2	10	5
	3	10	10
	4	-	-
	5	-	-
Klinefelter (Tannic)	1	160	80
	2	40	10
	3	10	10
	4	-	-
	5	-	-
Pregnancy (Kaolin)	1	40	80
	2	5	40
	3	-	10
	4	5	5
Pregnancy (Tannic)	1	20	20
	2	10	40
	3	5	10
	4	-	-
Menopausal	1	160	40
	2	10	5
	3	-	-
	4	-	-
	5	-	-
Pregnyl	1	10	40
	2	-	5
	3	-	-
	4	-	-
Gestyl	1	40	20
	2	-	10
	3	-	-
	4	-	-

Experiments on the Neutralising Power of Different Antisera.

Introduction.

The ability of an anti-hormone serum to neutralise or inhibit the biological effects of the corresponding hormone and/or other hormones, was the earliest immunological reaction of such antisera to be studied. Its importance derives not only from its significance for therapeutic work, but also from the fact that these reactions are obviously the most hormone specific reaction of antihormone sera. So much so that we have tried to shed more light on the specificity of the precipitation, complement fixation, and haemoagglutination reactions by comparing them with the neutralisation reactions.

Preliminary experiments were conducted to demonstrate the neutralisation by the antisera of endogenous and exogenous endocrine activity. During some of these experiments, instead of the inhibitory effect expected, antisera possessed the power to augment the

action of simultaneously administered hormone.

Preliminary Experiments.

1. Neutralisation and Antiserum Production.

In the first place the effect of the repeated injections of hormone extracts into rabbits for the production of antisera was observed.

Vaginal smears were made from each rabbit receiving injections of Klinefelter extract. The first smear was made before injection of the hormones and repeat smears were made at 3 day intervals for ten weeks. These were adult female rabbits, isolated from bucks. Since the rabbit only ovulates on coitus or possibly when in close proximity to the male the ovaries in these animals could be expected to be inactive and therefore no cyclical changes would be evident in the genital tract.

The rabbit ovary reacts to injected gonadotrophin by ovulating and producing steroid hormones. This was apparent in the vaginal smears taken during the days succeeding the

first few injections of gonadotrophin.

Prior to injection the smear showed a mixture of non-cornified and some cornified cells showing no tendency to clumping. Few leucocytes are present. (Fig. 2a). Figure 2b is a photograph of a smear taken three days after commencing injections. In this case there are sheets of cornified and pre-cornified cells. No leucocytes are present.

Several weeks later there was an absence of cornified cells with an excess of mucus and leucocytes. Figure 2c is one such smear made at the 10th. week of immunisation. This absence of cornification suggests that with repeated injections the hormone extracts had lost their effect on the gonads of the rabbit. While the foregoing results might be interpreted as a neutralisation of the hormone by antibodies other interpretations are possible. The ovary might go into a refractory state which had no immunological basis, and even if immunity were involved it could be of tissue

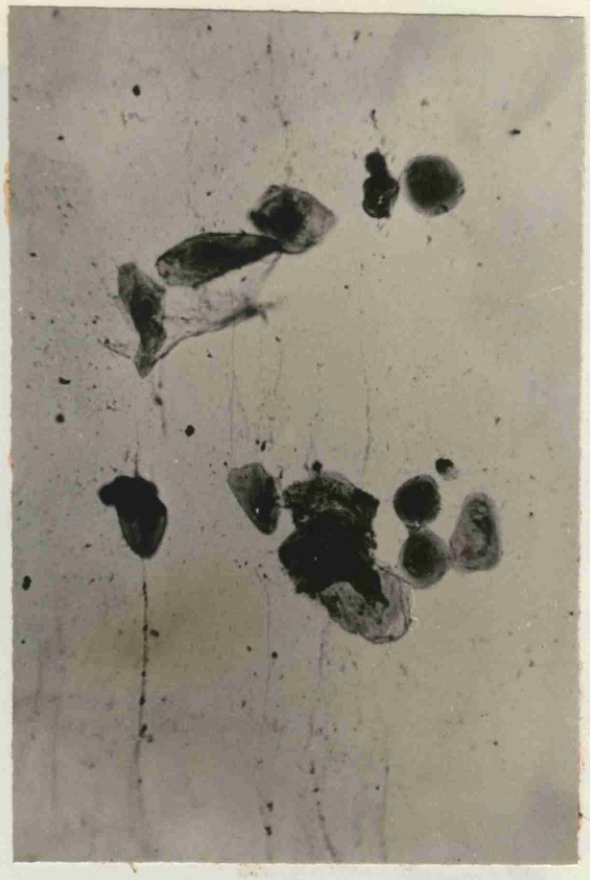


Figure 2.

Smear of rabbit at different stages of immunisation with a KF extract.
(Papanicolaou Stain).

A. Smear taken before starting the injections.
Mixture of intermediate cornified cells (dark) and basal non-cornified cells (pale).
X 125.

B. Smear taken five days after starting the hormone injections.
Excess of superficial and intermediate cornified cells.
X 125.

C. Smear taken after 10 weeks of continuous immunisation. Only pale (non-cornified) basal cells with mucus and leucocytes are seen.
X 125.

variety and not humoral. For those reasons further experiments were devised.

2. Neutralisation of Endogenous Gonadotrophin.

For the purposes of this experiment it was necessary to have an animal which has a natural and frequent ovarian cycle similar to that occurring in the human. For this reason adult female white wistar rats were chosen. These are a strain which has been inbred in the Royal Maternity Hospital, Glasgow, for the past 12 years and shown to have a regular 5 day cycle.

Three adult female rats were studied. Vaginal smears were made daily over a period to verify the sequence of changes. Following this each rat received 1 ml. of antiserum daily for 10 days (intraperitoneally). One rat received anti-pregnyl serum, one received anti-Klinefelter serum and the third anti-gestyl serum. These injections were begun in the oestrous phase. Daily vaginal smears were made during the period of experiment and for four days

following cessation of the injections.

Results.

The rat receiving anti-Pregnyl serum shows no departure from normal. The oestrous cycle proceeded normally through the phase of cornification, the whole cycle being completed in five days. Anti-Klinefelter serum seemed to prolong the cycle, the rat taking five days to achieve full cornification of the vaginal epithelium and a further two days to reach to oestrous phase.

In the case of anti-Gestyl serum the rat completed one normal cycle and thereafter the process was halted in the dioestrous phase for as long as the injections were continued. Shortly after cessation of injections the cycle was resumed and progressed normally.
Figures 3a,b,c, illustrate the normal oestrous phase prior to injection, the dioestrous pattern during the injection period and the resumption of the normal appearance two days after halting the injections.

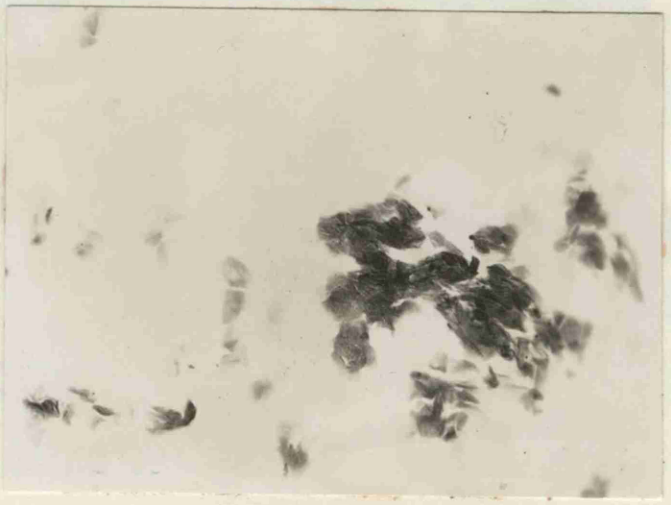
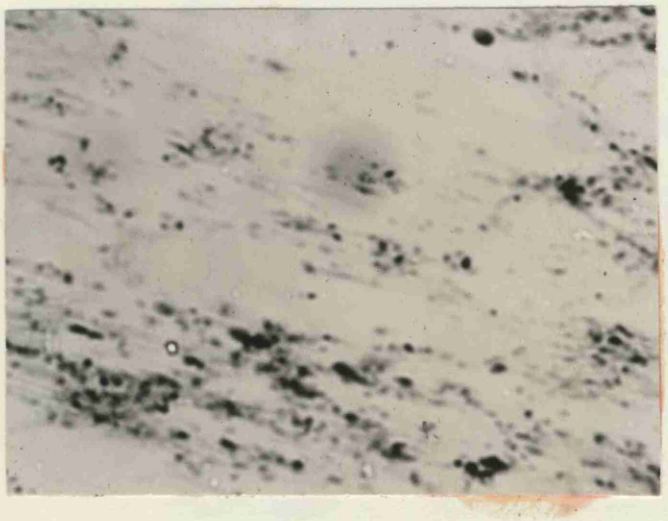


Figure 3.

Neutralisation of endogenous gonadotrophins of adult rat. (Papanicolaou Stain)
a) Vaginal smear taken one day before starting the injections of anti-Gestyl serum, showing 100% cornification. (oestrus).
X 125



b) Vaginal smear taken after three days of injection, showing abundant leucocytes, few non-cornified vaginal cells and no cornified cells at all (dioestrus). This picture persisted until the end of the treatment.
X125

c) Vaginal smear one day after stopping the antiserum injections showing the reappearance of cornified cells in considerable number.

X 125

3. Neutralisation of Exogenous Hormones.

The inhibitory as well as the augmenting effects which the antisera have on administered hormones were studied in three types of experiments. One preliminary experiment was conducted to study the rise and fall of the augmentation and the neutralisation powers in the sera of immunised rabbits. A second preliminary experiment was necessary to study the effect of changing the route of injection on those powers. Finally, the test proper was performed to study cross reactions between different antisera and different hormones. In those three experiments, two techniques were used to assess the results; the titration technique (Zondek and Sulman 1940), and the percentage inhibition technique. (Cole et al 1957).

Methods.

1. Neutralisation Titre of Antisera:

A quantity of the hormone antigen which contained approximately one mouse uterus unit,

was injected along with 1.5 ml. of varying dilutions of antiserum. The hormone was given in three divided doses to immature female mice, one dose daily for three days.

Half a millilitre of the appropriate serum dilution was also inoculated into each mouse daily for three days.

Four mice were used for each experiment. The antiserum was injected intraperitoneally, then two hours later the hormone was injected subcutaneously always making sure to raise a bleb.

To study the effect of the route of injection on the neutralising power of antisera, a number of experiments were performed, where some of the mice were injected as above, while others received both antiserum and hormone intraperitoneally, at two different sites with two hours interval (Cole et al 1957).

Three control batches were always used. One batch received only 1 m.u.u. of the hormone extract per mouse. The second batch received

1.5 ml. of distilled water per mouse. The third batch received only 1.5 ml. of antiserum per mouse.

The mice were killed on the 5th. day and their uteri were weighed. The highest dilution producing a 70-100 per cent inhibition of the increase of the uterus weight (in comparison to the increase of the uterine weight of the hormone control batch) was taken as the neutralising titre of the antiserum.

2. Percentage Inhibition.

A volume of 1.5 ml. of a 1/4 (or 1/8) dilution of each hormone extract tested was given to each mouse of the test batch and the hormone control batch.

The test batch also received 1.5 ml. of undiluted antiserum per mouse.

The routes of injection and the control batches were as in the first method.

The neutralising power of the antiserum was calculated according to the method of Cole et al (1957) from the following equation:

Inhibition Index (percentage inhibition) =

$$\frac{a-b}{a} \times 100$$

Where (a) is the increase of the mean uterine weight of the batch receiving only the hormone over the mean uterine weight of the batch receiving only distilled water.

And (b) is the increase of the mean uterine weight of the batch receiving both hormone and antiserum over the mean uterine weight of the batch receiving only distilled water.

When a prohormone effect was given by the antiserum instead of a neutralisation reaction, the augmentation index was =

$$\frac{b-a}{a} \times 100.$$

Results.

As previously stated during the early stages of production of antisera it was found that as well as containing antibodies the antisera possessed the power of augmenting

the activity of injected hormone. Table XXIV shows the behaviour of these antisera in this respect, the augmenting power being expressed in terms of dilution of the rabbit's serum.

Reference to this table shows that after one week of immunisation, the antiserum produced against Klinefelter extracts, even when diluted up to 1 in 8 augmented the effect of Klinefelter extracts on the mouse uterus. After 3 weeks a diluted antiserum (1 in 4 - 1 in 8) augmented, while undiluted antiserum neutralised such extracts. No augmentation could be detected after 4-5 weeks of continuous immunisation. When the injections were stopped the augmenting effect did not reappear, which indicated that it was not dependant on the amount of antibodies in the antiserum tested.

Table XXIV also shows that the same was true of other anti-hormone sera, including anti-pregnancy, anti-Pregnyl and anti-Gestyl, the latter retaining its augmenting powers

Table XXIV.

Rise and Fall of Augmentation Titre.

(Hormone injected subcutaneously).

Antiserum to	Antigen used for titration.	Rise of Titre					Fall of Titre.			
KFT	KF	8	4-8	0	0	Cessation of Immunisation.	0	0	0	0
CGT	CGT	4	16	0	0		0	0	0	0
Pregnyl	Pregnyl	8	8	0	0		0	0	0	0
Gestyl	Gestyl	16	32	0	0		0	0	0	0
Normal male.	KFT	N	2	0	0		0	0	0	0
Child	KFT	0	0	0	0		0	0	0	0
Interval in weeks.		1	3	5	10		1	3	5	10

KFT = Tannic acid extract of Klinefelter urine.
CGT = Tannic acid extract of pregnancy urine.
KF = Klinefelter urine.
N = Neat antiserum.

after 3 weeks of immunisation at a dilution of 1/32. Antiserum to normal male extracts showed slight augmentation effects when undiluted up to but not later than the third week of immunisation. Anti-child serum did not seem to possess such powers when injected subcutaneously in mice.

None of these antisera regained its augmenting powers after the injections were stopped.

The antigen used for the titration of anti-male serum and anti-Child serum was a Klinefelter tannic extract.

Cross Augmentation Reactions.

The various "early" antisera were tested for their power to augment antigens other than those used for their production, the 3 week period being chosen. The results are shown in Table XXV. Again the results are recorded in terms of dilution of antisera. It will be apparent that there was cross augmentation between all hormones and antisera tested.

Table XXV.

Cross Prohormone Reaction Titre.

(Antiserum and Hormone injected by different routes).

Hormone Extract	Antiserum to				
	KF	CG	HMG	Pregnyl	Gestyl.
KF	8	4	8	4	8
CG	4	4	4	4	8
Pregnyl	4	8	8	8	4
Gestyl	16	16	16	32	32
Normal male.	4	N	0	N	0
Child	0	0	0	0	0

KF = Klinefelter urine.
CG = Pregnancy urine.
HMG = Human Menopausal Extract.

Although the augmentation tended to be more effective when the antigen reacted with its own antiserum this was not constant and it was apparent that the reaction was neither species specific nor organ specific. Anti-Gestyl antisera to pregnant mare's serum possessed the highest augmentation power, and this was true for all hormones tested.

The influence of the route of injection on this activity was tested and in this case the augmentation index according to the method of Cole et al (1957) was calculated. Table XXVI is a recording of the results. In both experiments the antiserum was injected intra-peritoneally, while in one set the hormone was given subcutaneously and in the other intra-peritoneally. In every case augmentation was greater when both hormone and antiserum were injected intra-peritoneally, and even with biologically inactive extracts, such as from children, slight augmentation was obtained. Anti-Gestyl was the most potent of all,

Table XXVI.

Effect of Route of injection of hormone on augmentation index of sera.

Route:-	Subcutaneous Route.		Intraperitoneal Route.	
Hormone:-	KF	CG	KF	CG
Antiserum to				
KF	34	33	48	36
CG	38	42	44.3	51
Gestyl	54	51	76	64.8
Normal male.	22	16	25	20
Child.	0	0	5	7.4

KF = Klinefelter urine.
CG = Pregnancy urine.

producing a 76 per cent augmentation of Klinefelter extracts and a 65 per cent in the case of pregnancy extracts.

Titres of Neutralisation.

These are recorded in Table XXVII. In the case of anti-Klinefelter serum neutralisation was first obtained after 3 weeks of hormone injections but the serum was only active at neat.

This neutralising power was possessed by a 1/32 dilution of antiserum after 10 weeks of immunisation. Stopping the injections reduced the neutralising titre considerably down to 1/4 after 3 weeks. Slight neutralisation was detected 2 weeks later. It can also be seen that anti-Gestyl serum showed a power of neutralisation of Gestyl at dilutions of 1/8, 1/128, 1/256, after 3, 5 and 10 weeks of immunisation respectively. It had no such power after 1 week of immunisation, nor 10 weeks after the immunisation was stopped. Anti-CG and anti-

Table XXVII.

Rise and Fall of Neutralisation Titre.

Antiserum to	Antigen used for titration.	Rise of Titre.				Cessation of Immunisation.	Fall of Titre.			
KFT	KFT	0	N	8	32		16	4	N	0
CGT	CGT	0	4	16	32		16	N	N	0
Pregnyl	Pregnyl	0	4	16	64		16	4	0	0
Gestyl	Gestyl	0	8	128	256		32	8	N	0
Normal male.	KFT	0	0	N	N		0	0	0	0
Child	KFT	0	0	0	?N		0	0	-	-
Interval in weeks.		1	3	5	10		1	3	5	10

KFT = Tannic acid extract of Klinefelter urine.
CGT = Tannic acid extract of pregnancy urine.

anti-Pregnyl sera had neutralisation titres of 1 in 64 and 1 in 32 respectively after 10 weeks of continuous injections. Anti-normal male serum started to show neutralisation when undiluted at the end of the fifth week from starting the injections. Anti-child serum did not seem to have any neutralising powers, except occasionally when tested in undiluted form.

Both anti-male and anti-child sera were titrated against a Klinefelter tannic extract.

Cross Neutralisation Reactions, and their relation to the route of injection:

It can be clearly seen from Tables XXVIII and XXIX that the neutralising powers, like the augmenting powers of anti-hormone sera, are neither species-specific nor organ-specific. As a matter of fact, anti-serum to Gestyl (pregnant mare serum), was the most powerful neutralising antiserum when tested against extracts from Klinefelter, pregnancy, and menopausal urines and "Pregnyl",

Table XXVIII.

Cross Neutralisation Titre.

Hormone Extract.	Number of Experiments.	Antiserum to KF	CG	HMG	Pregnyl	Gestyl.
KF	12	32	8	32	8	16
CG	12	16	16	32	16	8
Pregnyl	8	8	4	16	8	16
Gestyl	12	128	128	64	128	256
Normal male.	6	4	N	2	N	0
Child.	3	2	N	N	0	0

KF = Klinefelter urine.
CG = Pregnancy urine.
HMG = Human Menopausal Extract.

Table XXIX.

Neutralisation measured as percentage inhibition.
(Hormone injected subcutaneously)

Antiserum to	Percentage Inhibition of:-				
	KF	CG	HMG	Pregnyl	Gestyl.
KF	95	63.4	72	18.3	31.6
CG	72.5	84	64	55.3	28
Pregnyl	35	44.5	29	31	23.7
Gestyl	100	98	100	82	76
Normal male.	37	13.8	21	19	0
Child	22	7	12.6	0	2
Normal rabbit serum.	0	-	-	-	-

KF = Klinefelter urine.
CG = Pregnancy urine.
HMG = Human Menopausal Extract.

all obtained from humans. Its neutralising power reached a titre of 1 in 256, and a percentage inhibition of 100%. It was more effective against pituitary hormones, (Klinefelter, menopausal), than against pregnancy urine extracts ("Pregnyl", pregnancy urine). Antiserum to the relatively cruder extract of pregnancy urine, showed higher titres than antiserum to the much purer extract Pregnyl (also from pregnancy urine). Sometimes, antisera were more effective against other extracts than against the corresponding extract.

When antiserum and hormone were both injected intraperitoneally, all antisera showed less neutralising powers, than when the hormone was injected subcutaneously. In the latter case, anti-child serum showed some neutralising effects, but no augmentation, while in the former case the opposite was true. This can be seen in Tables XXVIII, XXIX and XXX. These tables

Table XXX.

Effect of route of injection of hormone on neutralisation by antiserum.

Route:-	Subcutaneous Route (%age) Reaction.		Intraperitoneal (%age) Reaction.	
Hormone:-	KF	CG	KF	CG
Antiserum to				
KF	95	63.4	70	32.7
CG	72.5	84	66	78
Gestyl	100	98	82	71.5
Normal male	40	28	32	21
Child	22	7	5	0
Normal serum	0	0	0	0

KF = Klinefelter urine.
CG = Pregnancy urine.

also show that normal rabbit serum had no neutralising power at all, indicating that such power is not a non-specific reaction.

Conclusions on the Neutralising Effects of Anti-Hormone Sera:

The rise and fall of the anti-hormone powers in the sera of rabbits chronically injected with hormone preparations, suggest that they are due to the production of immune bodies, comparable to anti-enzymes. This is also supported by the absence of such powers from sera of untreated rabbits, or rabbits immunised with biologically inert extracts. This indicates that such powers are almost entirely specific in character and are not a non-specific activity of serum per se.

These neutralising antibodies seem to be neither species-specific nor organ-specific. That they are hormone-specific, is suggested by their abundance in antisera to highly active extracts, and their scarcity or absence in antisera to inactive extracts. The more

active the antigen, the more power of neutralisation its antiserum possesses.

Although anti-Oestyl serum had no power of precipitation, and slight power of complement fixation, it had the highest power of neutralisation. This suggests that the three reactions are not necessarily given by one and the same antibody.

Before the appearance of neutralising antibodies in an antiserum, some changes of obscure nature seem always to occur, imparting to this "antiserum" a prohormone activity. This change does not seem to be simply a change in the amount of circulating antibodies.

Absorption Experiments.

Anti-hormone sera were absorbed completely with inert and active extracts, and both the supernatants and the precipitates were studied. Such study was done to throw further light on points like the hormone specificity of the different reactions, the correlation of these reactions, and the nature

of changes in the serum proteins before and after reacting with different antigens.

To ensure reasonable antigen excess which is necessary for complete absorption of the antibody, two volumes of the optimal antigen dilution (1/4 to 1/8) were always added to one volume of undiluted serum. The mixture was incubated at 37°C in a water bath for one hour, then left overnight at room temperature. Next day, the mixture was centrifuged at 3000 r.p.m. for at least 15 minutes, until the supernatant was clear. The supernatant was then removed to a separate test tube and the precipitate was washed twice in ethanol and kept for testing. The supernatant was always tested against the absorbing hormone extract for precipitation reaction which if present would indicate that a second addition of antigen was required (Cruickshank & Currie, 1958).

Antiserum to Klinefelter extracts was absorbed with children-urinary extracts,

normal male extracts, pregnancy urine extracts and Klinefelter extracts. Antiserum to chorionic gonadotrophic extracts was similarly treated. The supernatants were tested for the titres of precipitation, complement fixation and neutralisation powers retained by them, as well as for their reactions in gel diffusion tests. The precipitates obtained from absorption were tested for their biological activity in immature female mice. The supernatants were also put on paper electrophoresis, along with normal rabbit serum and unabsorbed antisera as controls.

Precipitin and Complement Fixation Reactions of the Supernatant:

Reference to Table XXXI shows that when anti-Klinefelter serum was absorbed with child extract, the precipitin titre and complement fixation titre of the supernatant as tested with Klinefelter and pregnancy extracts, were about half the original titres

Table XXXI.

Absorption Experiments (Reactions of Supernatants).

Antiserum to	Absorption with	KF			CG		
		precip.	C.F.	Neut.	precip.	C.F.	Neut.
KF (Original titre of KF)		100	64	32	100	32	32
	Child	50	32	16	50	16	16
	Normal male	10	16	8	8	16	8
	CG	N	16	16	-	8	8
	KF	-	2	8	N	4	8
CG (Original titre of CG)		100	64	16	100	64	32
	Child	50	16	16	100	64	16
	Normal male	20	16	8	50	32	32
	CG	-	-	8	-	4	8
	KF	-	4	16	-	16	8

KF - Klinefelter urine.
CG - Pregnancy urine.
C.F. - Complement Fixation.
N - antiserum.

of the unabsorbed antiserum. Absorbing the same antiserum with Klinefelter or pregnancy extracts almost abolished the precipitin reaction and the complement fixation titre. The effect of absorption with normal male extracts lay half way between the effects of inert and active extracts.

Absorption of anti-pregnancy serum with similar extracts produced more or less the same type of results. For both antisera the supernatant seemed to react more strongly with the corresponding antigen than with the other inert or active extracts. This might indicate that the specificity of the supernatant is enhanced by absorbing some of the non-specific reactions of the original serum.

Neutralisation reactions of the supernatant:

Anti-Klinefelter and anti-pregnancy sera were absorbed with child, normal male, pregnancy and Klinefelter extracts, and each resultant supernatant was assayed for its power to neutralise Klinefelter and pregnancy extracts in

mice. For each type of assay, 16 to 20 mice were used, the hormone being injected subcutaneously, and the antiserum intra-peritoneally.

The information given in Table XXXI goes to show that the neutralising titre of the supernatants, was always lower than that of the original anti-hormone serum. After absorption with child extract, both anti-Klinefelter and anti-pregnancy sera had a neutralising titre of 1 in 16 when tested in mice against Klinefelter and pregnancy extracts, instead of original titres of 1/32. Anti-Klinefelter serum absorbed with pregnancy extract showed a titre of 1/16 against Klinefelter extract, and a titre of 1/8 against pregnancy extract, which suggests some degree of organ-specificity. However, anti-pregnancy serum absorbed with Klinefelter extract showed a higher titre against Klinefelter extract (1/16), than against pregnancy extract (1/8).

The above results would seem to suggest

that there is some definite relation between precipitation, complement fixation, and neutralisation reactions. What is more important is the fact that the biologically inert antigen or antigens in the childrens extracts, appear to be somehow, but definitely, related to the neutralising antibodies of antihormone sera. However, since complete absorption of antihormone sera only slightly affects their neutralising powers, it is possible that the two reactions are not due to one and the same antibody.

Biological Activities of Precipitates:

Each precipitate obtained from antigen-antibody mixtures in the absorption experiments, was resuspended in 12.5 ml. of distilled water, then injected intraperitoneally in four mice, each mouse receiving a total of 2.5 ml. Altogether, 8 mice were used for precipitates from Child extract and Normal Male extract, and 12 mice for precipitates from pregnancy and Klinefelter extracts, for each of the two

anti-hormone sera.

For the anti-Klinefelter serum, the average mouse uterus weights produced by the precipitates were 10 mg. for the menopausal precipitate, 7.9 mg. for the normal male precipitate, 14.6 mg. for the pregnancy precipitate, and 12.4 mg. for the Klinefelter precipitate. For the anti-pregnancy serum the values were 6.6 mg., 8.1 mg., 14.9 mg. and 13 mg. respectively.

It can therefore be seen from the results shown in Table XXXII that the precipitates retained a considerable proportion of the activity of the original extracts.

This table also shows that precipitates formed with anti-child serum, did actually, produce an increase in the mouse uterus weight, up to 60% of the control weight.

Electrophoresis of the Supernatants:

The supernatants resulting from the absorption experiments were electrophoresed on paper along with unabsorbed antihormone sera

Table XXXII.

Absorption Experiments. Biological activities of the precipitates.

Average increase in the mouse uterus weight as mg.. and % increase over the control.

Antigenic Extract.	original anti-extract.		ppte. with anti-CG		ppte. with anti-KF		ppte. with anti-child	
	mg..	% rise.	mg..	% rise.	mg..	% rise.	mg..	% rise.
CG	32	540	14.9	200	14.6	190	8.1	60
KF	24	400	13	160	12.4	148	7.25	45
HMG	21	300	8	60	10	100	7.6	50
Normal male	11	120	6.6	30	7.9	58	6.5	30

CG = Pregnancy urine.
KF = Klinefelter urine.
HMG = Human Menopausal Extract.

and normal rabbit serum. A volume of 0.06 ml. of each (all being a 1 in 3 dilution of the original sera) was applied with a pipette to the middle of a paper strip. Separation was carried out at a voltage of 100-120 for 16 hours at room temperature. The strips were then dried in hot air, stained in Naphthalene/ Black 12B, and scanned in a recording automatic scanner.

From Figure 4 it can be seen that immunisation of rabbits with hormone extracts produced a rise in all fractions of globulin including α, β and γ globulins.

Figures 5 and 6 show that absorption of antihormone anti-Klinefelter and anti-pregnancy sera with normal male extract diminished the amount of α and β fractions and to a less extent the γ - fractions while absorbing anti-hormone sera with hormone extracts, always reduced the amount of γ-globulins markedly.

Gel Diffusion Reactions of the Supernatants:

Some of the supernatants were tested in

agar Gel plates by the Ouchterlony technique. Details of the experiments and their results are given in the part on Gel Diffusion on page 165.

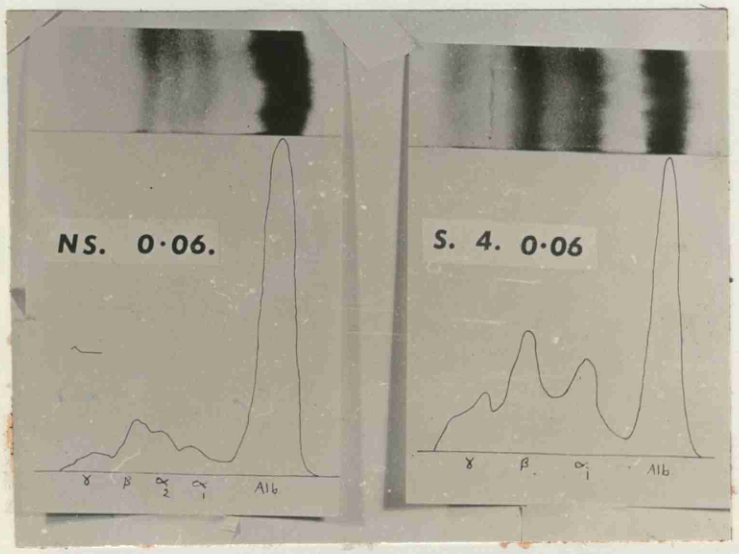


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Figure 4. Effect of immunisation (3 wks) on the globulins of rabbit's serum.
1. Serum before immunisation.
2. Serum after 3 weeks of continuous immunisation.

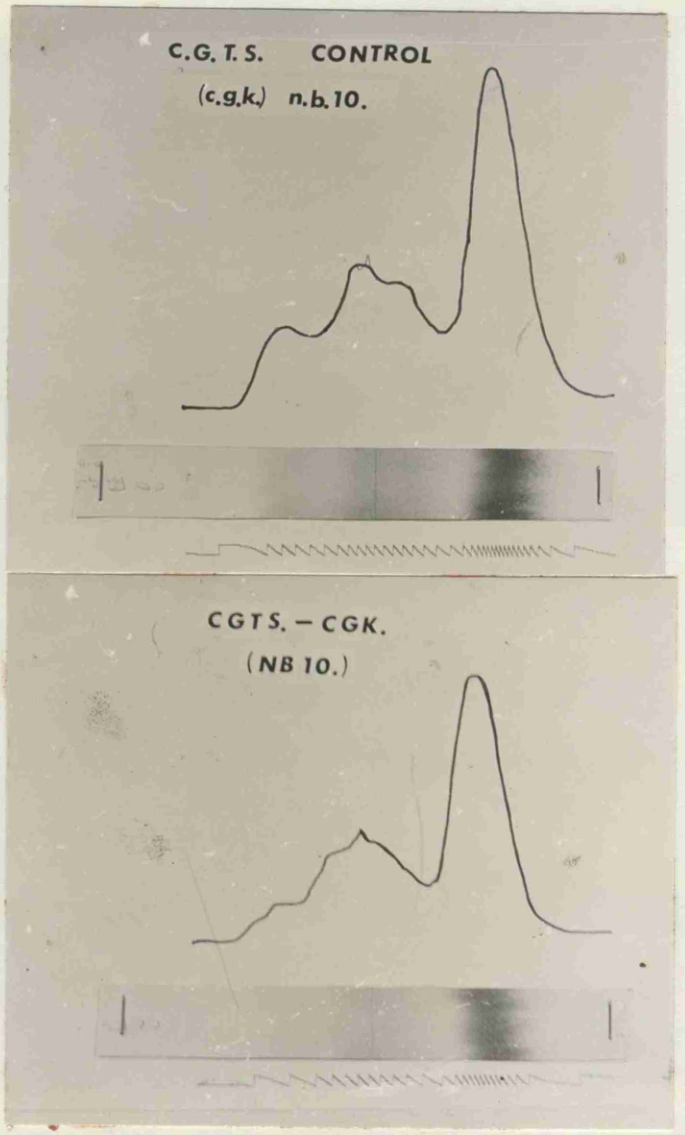


Figure 5. Effect of absorption on the globulins.
A. antiserum to tannic extracts of pregnancy urine CGT.
B. same antiserum after reacting with a kaolin extract of pregnancy urine CGK.

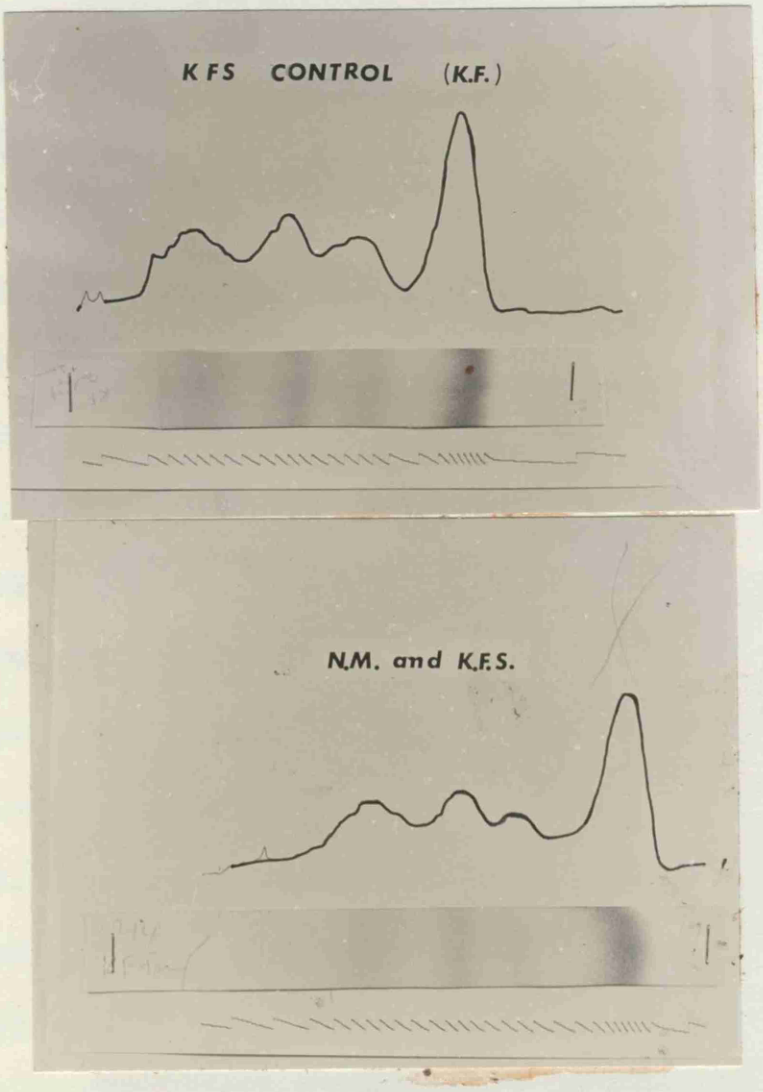


Figure 6. a and b. Effects of absorption on the serum globulins.

A. Antiserum to KF extracts.

B. Same antiserum after reacting with normal male extracts. Mainly α and β seem to be diminished.

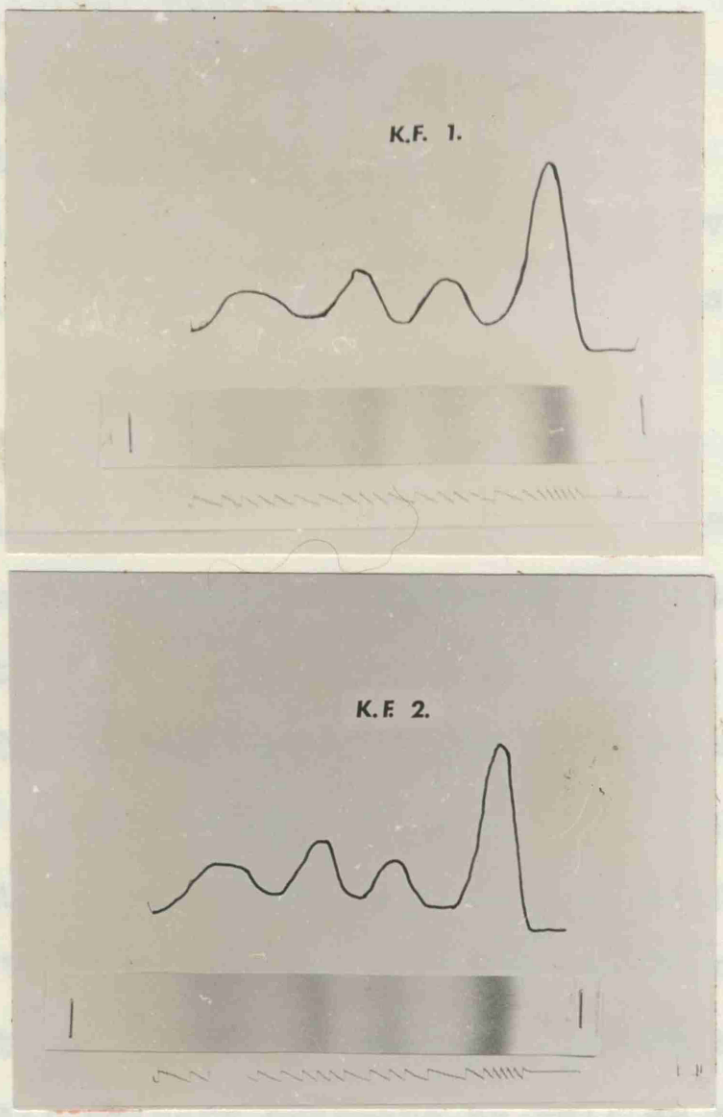


Figure 6. c and d. Effects of absorption on the serum globulins.

c. Same antiserum absorbed with a preparation of KF urine. γ-globulin seem to be diminished to a considerable degree.

d. Same antiserum absorbed with another KF extract. Same findings as in C.

General Summary and Conclusions of the Serological Tests.

Summary:

Types of reactions obtained:

1. All urinary extracts gave positive precipitin tests with their own antisera as well as other antisera.

2. The amount of precipitate, after maximum precipitation in the zone of slight antigen excess, proved as accurate as the titre of reaction for quantitative precipitin tests.

3. The hormone antihormone system required a long reaction time for maximum precipitation.

4. All urinary extracts as well as pregnant mare's serum extract gave positive complement fixation reactions with all the antisera used.

5. All urinary extracts as well as pregnant mare's serum extract gave positive haemoagglutination reactions with all the antisera. If these antigens were added to the antisera, the agglutination reactions were inhibited.

6. All the antihormone sera had the power to neutralise all exogenous hormones tested in mice. Anti-Gestyl serum was the most potent. It was also the only antiserum that inhibited the endogenous gonadotrophins of adult female rats.

7. Prolonged injection of rabbits with an extract of urine of a patient with the Klinefelter's syndrome (F.S.H. mainly) seemed to lose its effect after sometimes as shown by the changes in the vaginal cytology.

8. All antihormone sera showed at first prohormone effects and no neutralisation when tested against hormone extracts in mice. Later they augmented when diluted and neutralised when undiluted. Later still, they only neutralised and did not augment at any dilution.

9. Testing for neutralising and augmenting effects in mice, the route of injection affected them quantitatively but not qualitatively. An antiserum showed more

augmentation and less neutralisation when the hormone was injected intraperitoneally than when it was injected subcutaneously.

10. Antisera to inert extracts (e.g. child extract) showed slight augmentation (non-specific) only when the hormone was combined with it intraperitoneally.

Correlation of Serological Reactions and Biological Activities of Antigens:-

1. There was always discrepancy between serological reactions of an extract and its m.u.u. activity. Precipitin reactions showed the highest, and haemoagglutination inhibition reactions showed the lowest discrepancies. Complement fixation reactions showed more discrepancy than the direct haemoagglutination reactions.

2. Low activity and inert extracts were the source of most of the discrepancies. They usually gave strong reactions of precipitation and complement fixation, and positive reactions in Haemoagglutination and Haemoagglutination

Inhibition tests.

3. Neutralising effects were given only by antisera produced to active extracts. The more biologically active the antigen the stronger the neutralising powers of its antiserum (cf. anti-Oestyl).

4. Absorption experiments showed that the precipitates obtained from reactions between hormone extracts and antisera to active or inert extracts, always had some activity in mice.

5. The Haemoagglutination Inhibition test was specific for assaying and detecting the presence of chorionic gonadotrophin, only when the antigens tested were diluted urines or highly diluted extracts, but not concentrated extracts.

<u>The Effects of Purification on the Serological Reactions</u>:-

1. Purification of extracts produced lower titre of reaction in precipitation, complement fixation and haemoagglutination, but slight

change in haemoagglutination inhibition. The latter seemed least affected by impurities.

2. The discrepancy between m.u.u. activity and serological reactions was somewhat diminished but not abolished by purification of antigen or antiserum or both. Antigen purification was by itself more effective than purification of antiserum.

3. Pregnyl (benzoic acid preparation of Chorionic gonadotrophin) was weakly antigenic both in vivo and in vitro.

4. Low activity as well as high activity fractions obtained by ammonium sulphate purification of F.S.H. extract all gave strong precipitin reactions. The most active produced the strongest precipitation.

5. All biologically active fractions from Sephadex purification gave positive precipitation, complement fixation and haemoagglutination. The first fraction always gave the strongest precipitation even if it was not the most active biologically. The

haemoagglutination reactions showed more agreement with the biological activity.

Correlation of different Antibody Reactions:-

1. The titre of each antiserum was different for different reactions. The pattern of rise and fall of the titre varied from one type of reaction to another. (Fig. VII).

2. The effect of purification of the antigen on the strength of its serological reactions indicates that haemoagglutination inhibition depends on some factors different from those involved in complement fixation and precipitation.

3. Absorption experiments showed that after complete precipitation the supernatant antiserum showed negative or poor precipitation, considerable complement fixation, and strong haemoagglutination and neutralisation.

4. Neutralising antibodies are produced to only the biologically active extracts. Oestyl, which is highly active, produced antiserum with the strongest neutralising power,

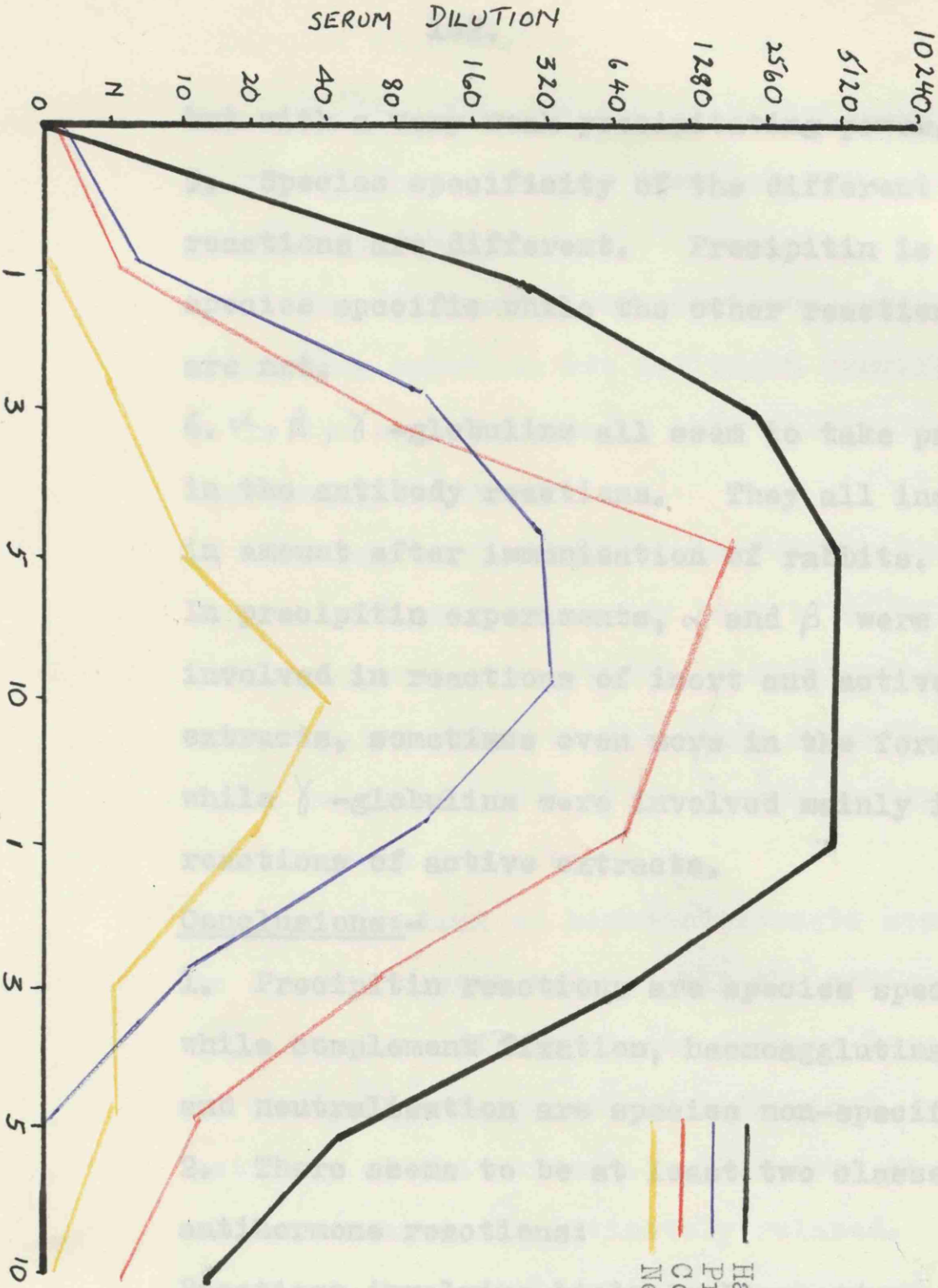


Figure VII. Rise and fall of titres of different reactions of KFT extract with its antiserum.

but with a very weak precipitating power.

5. Species specificity of the different reactions are different. Precipitin is species specific while the other reactions are not.

6. α, β, γ -globulins all seem to take part in the antibody reactions. They all increase in amount after immunisation of rabbits. In precipitin experiments, α and β were involved in reactions of inert and active extracts, sometimes even more in the former, while γ -globulins were involved mainly in reactions of active extracts.

Conclusions:-

1. Precipitin reactions are species specific while complement fixation, haemoagglutination, and neutralisation are species non-specific.

2. There seems to be at least two classes of antihormone reactions:

Reactions involving biologically active antibodies. These include neutralisation and haemoagglutination.

Reactions involving antibodies active only in vitro, these include precipitation and complement fixation.

The first class of reactions appears to be hormone specific but not organ specific, while the second class is only partially hormone specific.

3. The haemoagglutination inhibition test can be used as a pregnancy test by diluting out non-specific reactions. When using concentrated extracts fallacies arise from cross reactions with other gonadotrophins or antigenic impurities.

4. Purification of hormone extracts appears to eliminate some but not all the antigenic impurities. It also leads to some loss of the antigenic power and biological activity of the extract. Therefore the three changes seem to be intimately related.

5. It is impossible with any method of purification to prevent the biologically inert extracts from producing positive serological

reactions with antihormone serum.

6. The fact that absorption by biologically inert preparations of children's urine reduces the antibody reaction of an antiserum to pituitary gonadotrophins, particularly its neutralising power, suggests that there is something in children's urine chemically related to the gonadotrophins of pituitary origin.

7. The reduction of neutralising power of an antiserum by gonadotrophins originating in a different organ, indicates that the structure of all gonadotrophins is similar. The variations in degree of this reduction compared with that produced by absorption of the antiserum with its own gonadotrophin indicates, however, that there are minor variations in a structure.

8. None of the reactions seem to be organ specific. By organ specificity we refer to the different organs which produce the gonadotrophins, e.g. placenta and pituitary.

GEL DIFFUSION.

GEL DIFFUSION.

Introduction.

Oudin (1946, 1949) studied the precipitin reaction in gels, where he obtained a much better separation of multiple zones of precipitin, as well as better detection of faint zones, in a constant antibody titration. His technique was to allow the antigen to diffuse through a column of agar containing antibody (single diffusion or S.D.). Gels were also explored by Ouchterlony (1948, 1949, 1953), and Elek (1948, 1949) who devised a double-diffusion method in flat sheets of agar gel contained in Petri dishes, whereby the solutions of antigen and antibody diffuse towards each other from separate sources, (double diffusion or D.D.). As in Oudin's technique, lines of optimal precipitation occur, the number of distinct lines indicating the minimum number of different antigen-antibody systems present.

Wilson (1958) studied the relative merits of the two methods with respect to sensitivity, reaction range, reaction time,

resolving power and artefacts caused by temperature and concentration changes. He concluded that double diffusion is the better of the two in every respect.

The possible advantages of a qualitative analysis of the precipitin reactions of the hormone-antihormone systems are obvious. The lack of specificity of the immunological reactions, as well as the apparent failure of the chemical methods of purification, indicate that each extract used is a mixture of antigens. With gel diffusion techniques it may be possible to detect the number of such components in different types of extracts, to correlate some or all of these components with the biological activity in search for a specific reaction, and to demonstrate the effects of purification on the pattern of reaction of the hormone extracts.

Two more applications of the gel diffusion technique were tried. First, comparison of the pattern of reaction of some

extracts was compared with the pattern of reaction of some body fluids containing the hormones. This was to show the effects of chemical treatment on the antigenicity of the antigenic components. Second, the possibility of the presence of minute amounts of antigenic globulins in the extracts, was examined (Stacey and Barker, 1962).

Experiments.

The double diffusion technique of Ouchterlony was used to study the following problems:-

1. To demonstrate the patterns of reactions, cross reactions, reactions of identity between different antisera and hormone antigens.
2. To study the effects of purifying the antigens on the above reactions.
3. To study the effects of purifying the antisera on those reactions.
4. To investigate the reactions of the biologically inert extracts.
5. To test the reactions of body fluids

(urines and sera) in gel diffusion.

6. To study the effects on the reactions of absorbing the antisera with different antigens.

7. To study the immunological relationship between anti-human-globulin serum (Coomb's reagent), hormone preparations and hormone antisera.

The reactions were always examined for the following points:

a. Number of lines produced.

b. Relative intensity of the lines.

c. Reactions of identity, non-identity and partial identity.

Material.

Several antigens were tested in gel diffusion, some of them crude and some purified. Antisera to crude and purified extracts were similarly tested.

a. Crude antigens.

Kaolin extracts were prepared from the urine of several Klinefelter patients, pregnant women, postmenopausal women, normal

males and normal females. At the same time kaolin extracts with no biological activity were obtained from the urines of children and a previously hypoxed male (342). Similar inert extracts were obtained by altering the pH of extraction of urine from Klinefelter patients, pregnant women and postmenopausal women.

b. Purified antigens.

Tannic extracts were prepared from urine of Klinefelter patients, pregnant women, postmenopausal women, normal males, normal females and children under the age of five. Proprietary benzoic acid extracts of pregnancy urine were also used (Pregnyl Organon). An ion exchange extract of postmenopausal urine, obtained from Johnsen (J3), was used in some experiments.

A benzoic acid extract of Pregnant Mare Serum (Gestyl Organon) was used to study the species specificity of the reaction.

Kaolin extracts further purified by

salt fractionation (S.A.S. method of Johnsen 1955), kaolin extracts purified by adsorption on tricalcium phosphate (Loraine Brown and/(954). were also tested. Finally, the fractions obtained by gel filtration on Sephadex G-25, of kaolin and tannic extracts of Klinefelter and pregnancy urine extracts were examined by the Ouchterlony technique.

c. Crude antisera.

Antisera to kaolin extracts of Klinefelter urines, pregnancy urine extracts and children's urine extracts were used.

d. Pure antisera.

Antisera to tannic extracts of Klinefelter patients' urine, pregnancy urine and children's urine were used, and considered much purer than antisera to kaolin extracts.

Antiserum to Pregnyl was used as the purest antiserum available to us. Also antiserum to Gestyl extracts were tested for the species specificity of the gel diffusion reactions.

Finally, the γ -globulin fraction of several antisera was obtained by salting out, as an attempt at further purification of the antisera.

Method.

The Ouchterlony technique, as used by Cruickshank and Currie (1958), and Henry and Van Dyke (1958), was applied. The central cup was filled with the antiserum, and the peripheral cups were filled with the antigens. One addition was resorted to. The plates were allowed to stand at room temperature.

Results.

Preliminary experiments:

1. Frequency of application of reagents:

If more than one addition of antigens or antiserum was resorted to, a large number of lines appeared in place of the few lines that show after a single addition of each of the reagents. (Figure 14).

2. Temperature of reaction.

If the reaction was allowed to take place at room temperature, lines usually appeared within three days. Putting the plates at 4°C, did not allow the lines to show before the end of 5 to 7 days. Incubating the plates at 37°C. only slightly accelerated the appearance of the lines, which took 2 to 3 days. However, in the latter case, infection of the plates with anthracoids and fungi was high.

3. Reaction time:

Sometimes multiple lines of reaction would appear as early as the third day. More often, a single line reaction or no reaction at all was obtained after such period. The reaction was read at 2 to 3 days intervals. After five days, a single line reaction was usually suitable for photography. After seven days, more lines appeared, but not all of them were strong enough to show in a photograph. More lines appeared and/or the early lines became stronger until 10 to 21

days of reaction after which period the reactions remained stationary.

Reference to figures 3a and 3b illustrates the change in the picture of the reaction with time.

Patterns of reactions.

From our experiments, it is quite obvious that the reactions of kaolin extracts with antisera to kaolin extracts produced not one, but several lines indicating the presence of several antigens, and therefore several antibodies in these extracts and their antisera. From figures 1 and 4 it can be seen that Klinefelter extracts, not only contained more than one antigen, but also the number of antigens in each Klinefelter extract was different from the number of antigens in other Klinefelter extracts. However, a dominant pattern of two strong lines and a third weaker line can be seen.

Similarly, testing a Kaolin extract of pregnancy urine against its own antiserum (figure 3b), shows the presence of more than

one antigen in such extracts. The main pattern consisted of two strong lines which seemed to be identical with two of the lines produced by Klinefelter extracts.

Normal male extracts produced two fairly strong lines, joining with identical lines of the Klinefelter extracts. Normal female extracts tended to form two lines identical with lines produced by Klinefelter, pregnancy urine and normal male extracts.

Postmenopausal extracts almost invariably produced a single strong line, identical with the middle line of the Klinefelter extracts as well as with lines given by all the other extracts.

Effects of purification of the antigens on the pattern of reaction: (using antisera to crude kaolin extracts).

From figure 4 it can be seen that the purer tannic extracts of Klinefelter urines, generally produced less intense lines than their kaolin counterparts. However, the

number of lines remained essentially the same. Similarly, figure 8 shows that tannic extracts of pregnancy urine, produced two lines exactly similar to those produced by kaolin extracts.

The same findings were true of tannic extracts of male urine, female urine and postmenopausal urine.

Treatment of kaolin extracts with tricalcium phosphate gel, or the omission of such treatment did not seem to affect the intensity or the pattern of reaction of Klinefelter or Pregnancy urine extracts (figure 5), or any other extract. Although the gel appeared to remove some of the ingredients of those extracts, it did not seem to remove any of the antigens.

Filtration of kaolin and tannic extracts of Klinefelter, Pregnancy and postmenopausal urine on Sephadex columns, led to the separation of each extract in several fractions. Only the first fraction of each extract

produced any reaction in gel diffusion. Although the original Klinefelter extracts, both kaolin and tannic gave two to three lines, fraction 1 gave only one strong line, in the case of either extract (figure 6). Similarly, reference to figure 9, shows that of the Sephadex fractions of both kaolin and tannic extracts of pregnancy urine, only the first one of each gave a reaction, which consisted of two lines.

Pregnyl (benzoic extract of pregnancy urine), produced two lines, weaker in intensity but identical with those of other pregnancy extracts. One of the lines showed reaction of identity with the middle line of Klinefelter extracts. (Figures 12 and 14).

Figure 7 shows that the original kaolin extract of Klinefelter urine produced one strong line, and two fainter lines: an inner line closely adjacent to the main line and an outermost line. Salt treatment of the mother substance (S.A.S. method), did not abolish any

of the lines, which appeared in all the fractions which possessed some biological activity viz. fractions 1, 55, 70 and 100. However, the two inner lines became more widely spaced. The third outermost line appeared distinctly in fraction 55, which possessed high biological activity. There also seemed to be a faint similar line produced by fraction 70, the other highly active fraction.

The ion exchange extract of postmenopausal urine (J3), took a long time to produce one main line reaction in gel diffusion, of rather low intensity. (Figure 12).

The Effect of Purification of antisera on the pattern of reaction: (using crude and purified extracts).

Antiserum to tannic extracts of Klinefelter urine was allowed to react with kaolin and tannic extracts of this urine. It was found that the reaction between the purer antiserum and the crude kaolin extracts was more or less

the same as the reaction between the cruder antiserum and the same extract. Also the reactions of tannic extracts of Klinefelter's urine with antisera to such extracts seemed to be identical with the reactions occurring between kaolin extracts and the same antiserum (figure 10).

Figure 3 b also shows that the reactions of both tannic and kaolin extracts of pregnancy urine seem to be identical. The innermost line gives a reaction of partial or of non-identity with the Klinefelter lines.

With all the other extracts, a purer antiserum produced the same pattern of reaction and number of lines, often but not always, with a weaker intensity.

The reactions of the antiserum to Pregnyl (benzoic extracts of pregnancy urine), were so weak that the plates could not be photographed. However, the patterns of reaction were the same as those of other antisera to pregnancy extracts. Kaolin and tannic extracts of pregnancy urine produced

a two line reaction. Kaolin and tannic extracts of Klinefelter urine, each produced a three line reaction. The middle line was identical, the inner line non-identical with lines produced by pregnancy extracts.

Using the γ-globulin fraction of crude antiserum to kaolin extracts of Klinefelter's urine, instead of the whole antiserum, indicated that the γ-globulins possess most of the antibodies involved in the gel diffusion reactions. It also suggests that the use of γ-globulins of antisera instead of whole antiserum, does not eliminate any lines or make the reactions more specific. Figure 11 shows that, at the end of 14 days, kaolin and tannic extracts of Klinefelter urine gave two strong and one weak line. Kaolin and tannic extracts of pregnancy urine each produced two lines, an outer strong line identical with the middle line of Klinefelter and normal male extracts, and an inner much fainter line, identical

with the faint innermost line of the pituitary hormone extracts.

Species Specificity of gel diffusion reactions: (Figures 14 and 20).

Pregnant mare serum extracts (Gestyl) never produced any lines when allowed to react with antisera to Klinefelter extracts or pregnancy urine extracts. Anti-Gestyl serum, also never produced lines against any human extracts, whether these extracts were biologically active or inert. However, Gestyl produced two faint lines when allowed to react for a rather long time, with its own antiserum.

It was therefore concluded that gel diffusion reactions were strictly species specific. Therefore, although gel diffusion is supposedly much more sensitive than interfacial precipitation or microprecipitation technique, yet it still confirms the absence of any cross precipitation between human and horse gonadotrophins.

Organ specificity of gel diffusion reactions:

It is quite obvious from the above experiments that, although precipitins in gel diffusion are apparently species specific, they are far from being organ specific. Cross reactions occurred among extracts of Klinefelter urine, postmenopausal urine and pregnancy urine, as well as their antisera. The purification of antigens or antisera by any technique, did not hamper in any degree such cross reactions. This is obvious from the figures already mentioned and is further illustrated in figure 12.

Hormone specificity of gel diffusion reactions:

Several findings suggest that the lines of reaction in gel diffusion experiments are, at least partly, related to the hormone activity of the extracts tested. However, other findings cast some doubts on this hormone specificity.

That these reactions are hormone specific seems to be supported by the following:

1. Each biologically active extract seemed

to possess a more or less constant pattern of reaction. Purification of such extracts by any of the methods tried, did not change the patterns. The number of lines and their reactions of identity remained the same, although their intensities sometimes diminished. Figure 7 shows that the lines of the S.A.S. fractions of a Klinefelter extract, remained identical with the lines of the mother substance.

2. More often than not, the extracts with high biological activity produced stronger lines, than did the extracts with low or no biological activity. From figure 7 it can be seen that the strongest lines were produced by the mother substance and the two most active fractions (55 and 70), and the weaker lines by the less active fractions (1 and 100). Furthermore, when the biological activity completely disappeared (fraction V), no lines were produced.

3. Gel filtration of Klinefelter and pregnancy urine extracts on Sephadex columns, resulted

in 4 to 5 fractions for each extract. However, gel diffusion reactions were produced only by fractions which possessed considerable part of the biological activity of the mother substance. (figure 9).

Extracts of postmenopausal urine produced only one fraction with biological activity after gel filtration. This also was the only fraction to give gel diffusion reactions. This becomes significant when we remember that the usual pattern of human menopausal extracts was always a single line of reaction. It is highly probable that this single line corresponds to the single Sephadex fraction, which carries all the biological activity.

The following findings however, throw some doubt on the specificity of these reactions.

1. Biologically inert extracts always produced considerable reactions, which were often identical with lines produced by

biologically active extracts. Figures 16 and 20 show how children's urine extracts, extract of urine from a hypoxed male, produced strong lines with reactions of identity with lines of hormone extracts. Biologically inert extracts of pregnancy urine due to storage (Pregnyl), and biologically inert extracts of urine from Klinefelter patients, pregnant women and postmenopausal women, extracted at unusual pHs., gave reactions identical with those of their biologically active counterparts. (Figures 10, 17).

2. Extracts of low biological activity did not always give reactions of less intensity than those of highly active extracts, (figure 16).

3. Antisera to biologically inert extracts, like extracts of children's urine, produced considerable cross reactions with biologically active extracts. Although, such antisera produced identical lines of reactions with inert and active extracts (figure 10), yet the

intensity of the reaction was usually more with the highly active extracts (e.g. HCG and Klinefelter extracts). This indicates that the reactions of antisera to children's extracts are somehow related to the biological activity of the hormone extracts.

4. The species specificity and the lack of organ specificity of these reactions may be thought to indicate that they are not hormone specific. However, this could be due to genuine antigenic difference between human and animal hormones, or this could be due to the actual nature of hormone precipitating antibodies which may be quite different from antibodies involved in other hormone reactions.

Contaminating antigens:

It has been shown electrophoretically that gonadotrophic extracts may contain minute quantities of albumin, not detected chemically (Johnsen 1961).

We tried to investigate this possibility

immunologically by comparing the reactions of healthy urines and urines showing gross proteinuria by chemical tests. Such extracts were prepared from urines of Klinefelter patients, pregnant women, and children under the age of five.

Extracts of urines showing proteinuria gave identical patterns, number of lines and intensity of reactions as those of the normal counterparts. Purification of any of the former extracts, by tannic acid, tricalcium phosphate or on Sephadex, did not make any difference to their reactions in gel diffusion. This seems to indicate that plasma proteins (especially albumin), cannot form a major antigenic component of our extracts.

The possibility of the presence of minute quantities of globulins in hormone extracts was further investigated, due to the physico-chemical resemblance of some globulin fractions to serum glycoproteins,

including gonadotrophins (Stacey and Barker, 1962). Antiserum to total plasma globulins (Coomb's A.H.G. reagent), was allowed to react with biologically active and biologically inert extracts. Anti-hormone sera were tested simultaneously, as a control. It can be seen from figure 15, that A.H.G. produced a weak line of reaction with Klinefelter extracts, which was not identical with the main lines of reaction produced by anti-Klinefelter serum against the same extract. The same findings obtained with pregnancy urine extracts.

Extracts of children's urine showing heavy proteinuria, did not give stronger reactions against A.H.G., than extracts of healthy children's urine.

These findings again confirm that globulins are only minute irrelevant contaminants of our hormone extracts.

Absorption of antisera:

Preliminary experiments showed that

more than one addition of antigen was required for the complete absorption of antisera to gonadotrophins. After such absorption, no precipitate was obtained when the supernatant was tested against the absorbing antigen by the interfacial or micro-precipitation techniques. However, such supernatant always gave some reactions in gel diffusion, apparently due to small quantities left over, and not detectable by the less sensitive tests. This natural tendency of the gonadotrophin-antigonadotrophin system was very convenient, since it was possible by complete absorption to remove the weaker antibodies from an antiserum and leave behind enough of the stronger antibodies to be examined in gel diffusion.

When antisera to Klinefelter extracts were absorbed with kaolin or tannic extracts of Klinefelter urine, the supernatant did not produce lines, until the end of two weeks. When the lines appeared, they did so first

with HCG then with Klinefelter extracts.

The same results were obtained when antisera to pregnancy urine extracts were absorbed with kaolin or tannic extracts of pregnancy urine.

Since the first extracts to show reaction with the supernatant was also the most active biologically, (HCG), it is reasonable to consider that the minute amount of antibodies left over after absorption were related to the hormone activity.

After a suitable length of time, biologically active and biologically inert extracts showed reactions with the absorbed antisera. The reaction was always a single line identical in all of them. However, the lines produced with HCG, Klinefelter extracts and HMG, were stronger than those produced by normal male and normal female extracts.

Antisera to kaolin and tannic extracts

of Klinefelter and pregnancy urine were also absorbed with kaolin and tannic extracts of children's urine extracts. Given enough length of time, the supernatants produced reactions with hormone extracts as well as with children's urine extracts. However, the lines produced with the biologically active extracts were stronger than those produced with the biologically inert extracts. (Figure 19).

From the above, it is seen that although absorption of antisera succeeds in producing single line reactions, yet it does not prevent biologically inert extracts from reacting with the antihormone sera. Thus, although on one hand the gel diffusion reactions appear to be related to the hormone activity, yet on the other hand they seem to depend on the presence of antigenic substance or substances common to the hormone extracts and the children's urine extracts as well as to other biologically inert extracts.

Reactions of body fluids in gel diffusion.

Using antiserum to kaolin extracts of Klinefelter urine:

Fresh sera from pregnant women and sera from non-pregnant women, each produced reactions of three identical lines. Fresh urine from pregnant and non-pregnant urine produced one identical line, which was also identical with the middle line in the reaction of the sera.

Using antiserum to tannic extracts of Klinefelter urine:

The reactions as a whole were less intense than those of the above experiment. However, the sera of pregnant and non-pregnant women still produced identical reaction of three lines each, and urine of pregnant and non-pregnant women produced identical reactions of a single line each.

Using antiserum to kaolin extracts of pregnancy urine:

Fresh sera of pregnant and non-pregnant women produced identical reactions of three

lines each. Fresh urine from pregnant and non-pregnant women produced an identical single line reaction.

Using antiserum to tannic extracts of pregnancy urine:

The reactions were the same as those in the above experiment, only somewhat less intense.

Summary:

The single line reaction in gel diffusion is not a reliable criterion of the chemical purity of the extracts, since it depends mainly on the relative concentration of the antigenic components, and hence on the time allowed for the reaction. It is therefore quite clear that, both crude and purified extracts contain multiple antigenic components. From the S.A.S., and Sephadex fractionation methods, it appears that the biological activity is actually related to more than one of these antigenic components. Purification of extracts does not remove any of the components but only removes parts of each component at the same time. Therefore, although

conceivably we could reach a stage of purification where almost only the main antigen is retained in the extract, yet we must expect a definite loss of some of the total activity in the process of purification.

It is also clear, that children's extracts (and other inert extracts) and antisera to such extracts, react in gel diffusion due to the presence of some antigenic component, immunologically and probably chemically identical with the antigens involved in the reactions of hormone extracts and their antisera. This antigenic component or components could conceivably be a hormone carrier, excreted in human urine whether the hormone was there or not.

The single line of reaction produced by fresh urines in gel diffusion may suggest that the multiplicity of the antigens in the extracts is the result of the chemical processes of extractions. On the other hand, the difference can be explained as due to

the presence of one strong antigen in fresh urine with other much weaker ones. The extracts of course concentrate the weaker antigens along with the strong ones. This is substantiated by the multiple lines produced when fresh human sera reacted as antigens with anti-hormone sera. This also suggests why haemoagglutination tests can be successful in the diagnosis of pregnancy only when fresh diluted urine is used, but not when concentrated extracts were tested.

Figure 1. Central cup contains antiserum to a Klinefelter urine extract M. A, R, and M are urinary extracts from three Klinefelter patients.

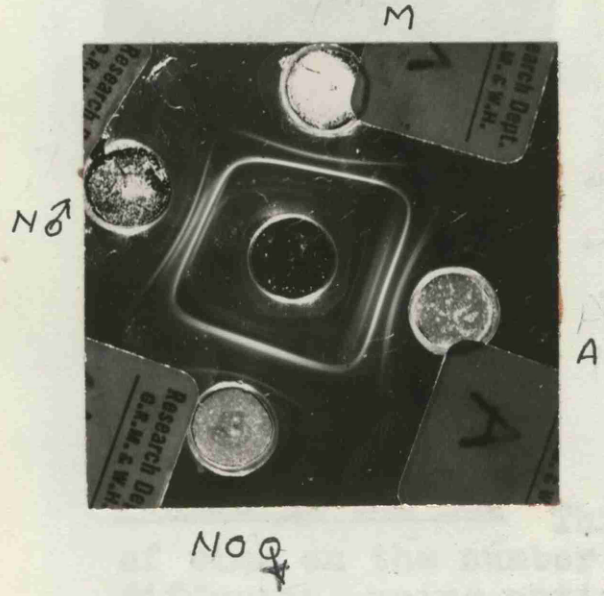
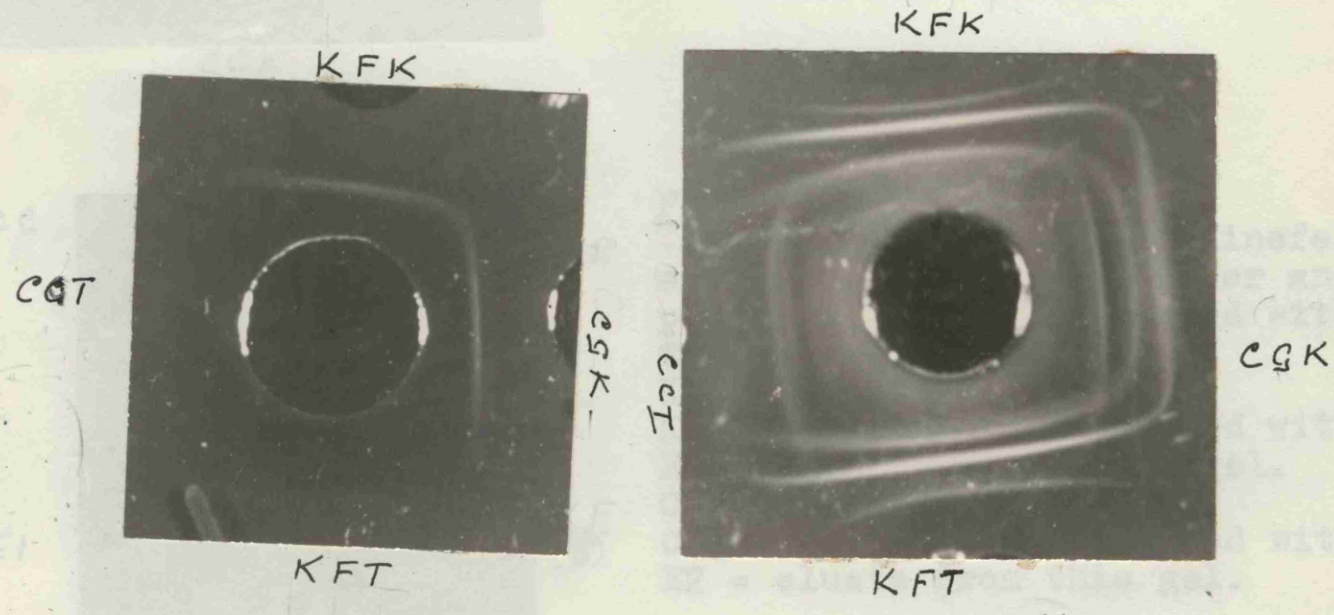


Figure 2. Antiserum to Kaolin extract of Klinefelter urine. A and M are two Klinefelter extracts. N♂ = normal male extract. N♀ = normal female extract.



KFK = Kaolin extract of kline-felter urine
KFT = Tannic extract of klinefelter urine
CGK = Kaolin extract of pregnancy urine
CGT = Tannic extract of Pregnancy urine

Figure 3a and 3b. This illustrates the effect of time on the number of lines obtained in gel diffusion, using antiserum to tannic extracts of pregnancy urine. Note the multiplicity of lines, both with crude and purer extracts, and the cross-identity between lines in pregnancy and Klinefelter extracts.

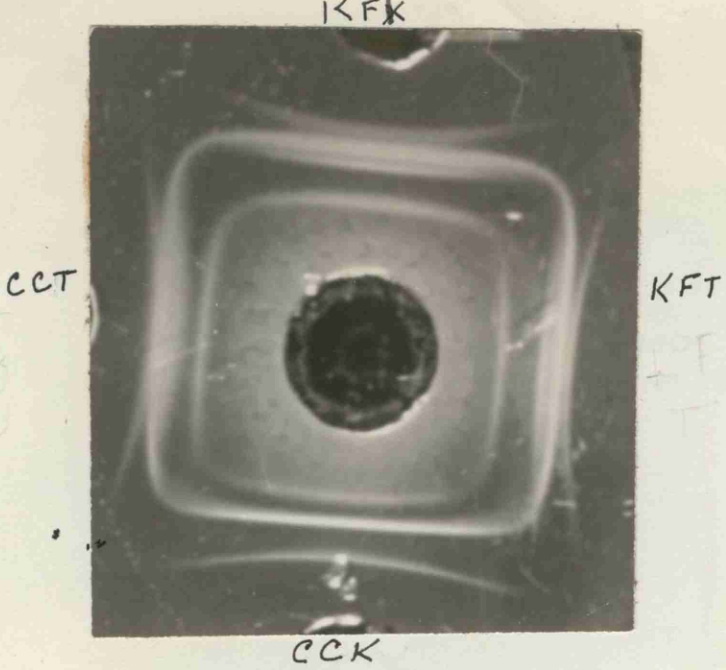


Figure 4. This illustrates clearly that purification of the antigens produces no change in the number of lines. Antiserum to Klinefelter extract used against Kaolin and tannic extracts of Klinefelter and pregnancy urines.

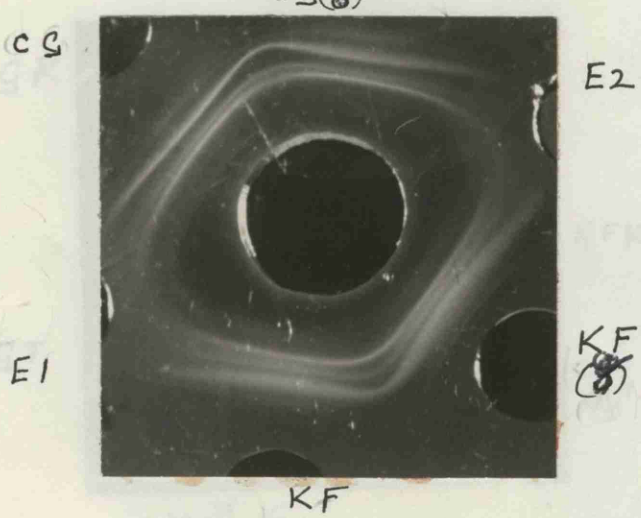


Figure 5. Antiserum to Klinefelter extract against Klinefelter and pregnancy extracts treated with tricalcium phosphate gel.
KF = Klinefelter extract.
KFg = same extract treated with gel.
E.I. = eluate from this gel.
CG = Pregnancy extract.
CGg = same extract treated with gel.
E2 = eluate from this gel.

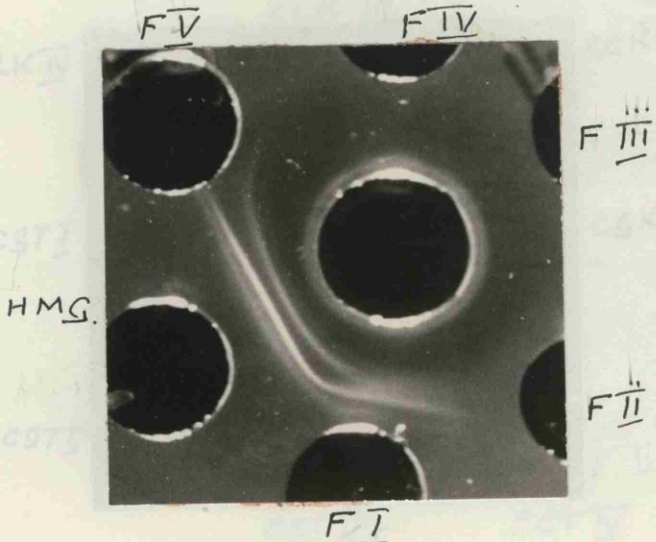


Figure 6. Antiserum used against fractions separated by Sephadex from a menopausal extract. Note that the first, the only biologically active one is the fraction which produces a reaction but the lines are still multiple.
(Antiserum to Klinefelter extracts was used).

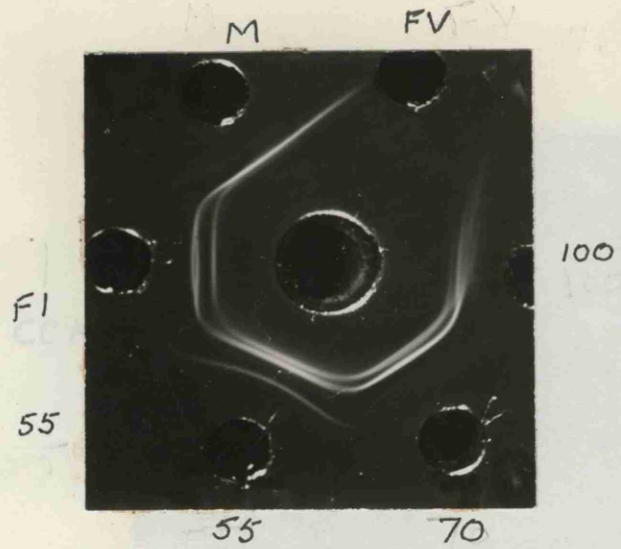


Figure 7.

Klinefelter antiserum V. fractions obtained from Klinefelter extract by ammonium sulphate treatment. M = original extract. F1 = first fraction. 55 = precipitate at 55% saturation. 70 = precipitate at 70% saturation. 100 = precipitate at 100% saturation. FV = final supernatant. Separation of lines is not as good as usual. Note lines are produced by active fractions and are multiple.

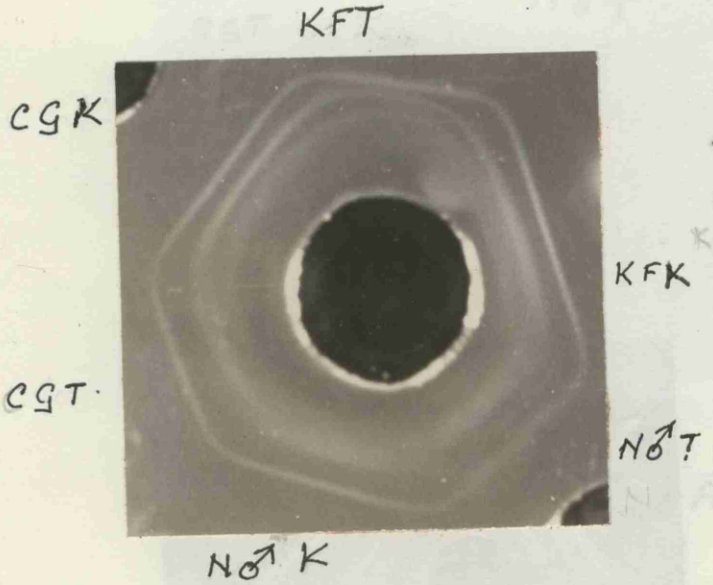


Figure 8.

Antiserum to pregnancy extract V. Kaolin and tannic extracts of pregnancy, Klinefelter and normal male urines. The reactions are similar with both crude and purer extracts and there is identity of lines in all extracts.

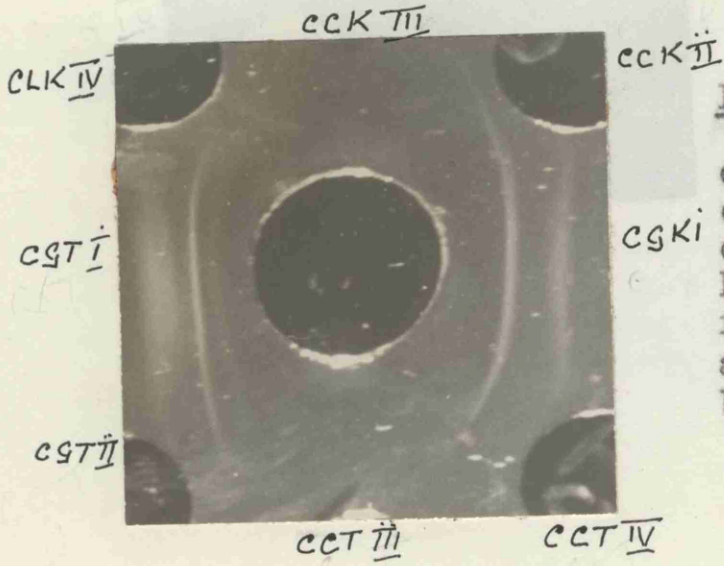


Figure 9.

Antiserum to pregnancy extract V. Sephadex fractions obtained from Kaolin and tannic extracts of pregnancy urine. Note that in both instances only fraction I gives a reaction, although fraction II was also biologically active.

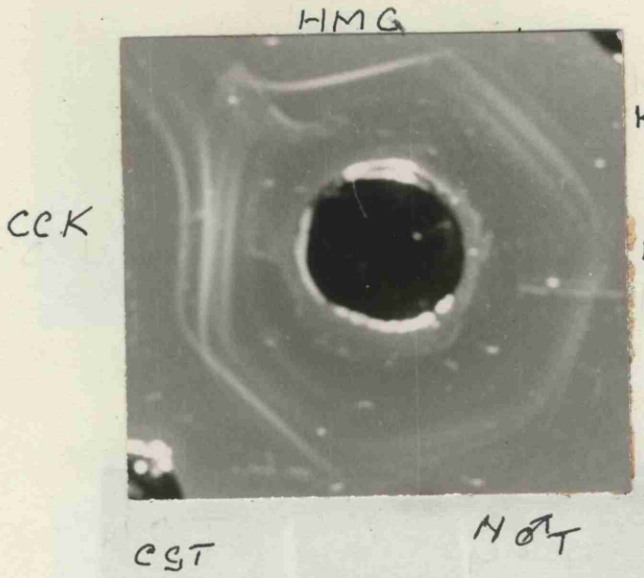


Figure 13. Tannic Klinefelter antiserum V. Kaolin and tannic extracts of Klinefelter (KFK, KFT) and pregnancy (CGK, CGT) urines, tannic extracts of male urine (N♂T) and menopausal urine (HMG). Tannic extracts give weaker reactions and some of the lines in menopausal and pregnancy extracts are non-identical.

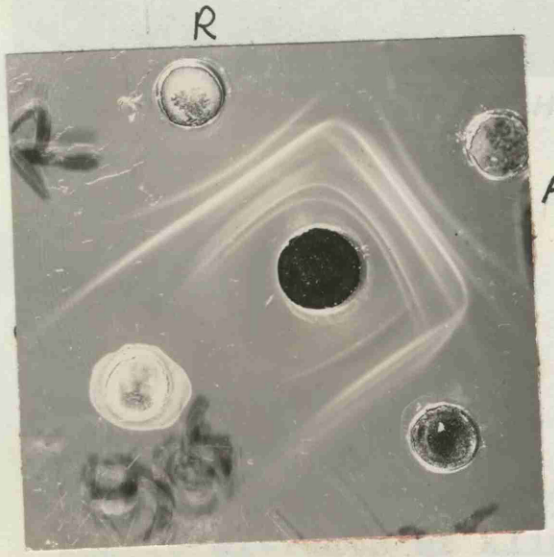


Figure 14. Klinefelter antiserum V. two Klinefelter extracts (A,R), Pregnyl (P) and Gestyl (G). Note lack of reaction with Gestyl.

(Reagents were applied more than once which led to splitting of the lines).

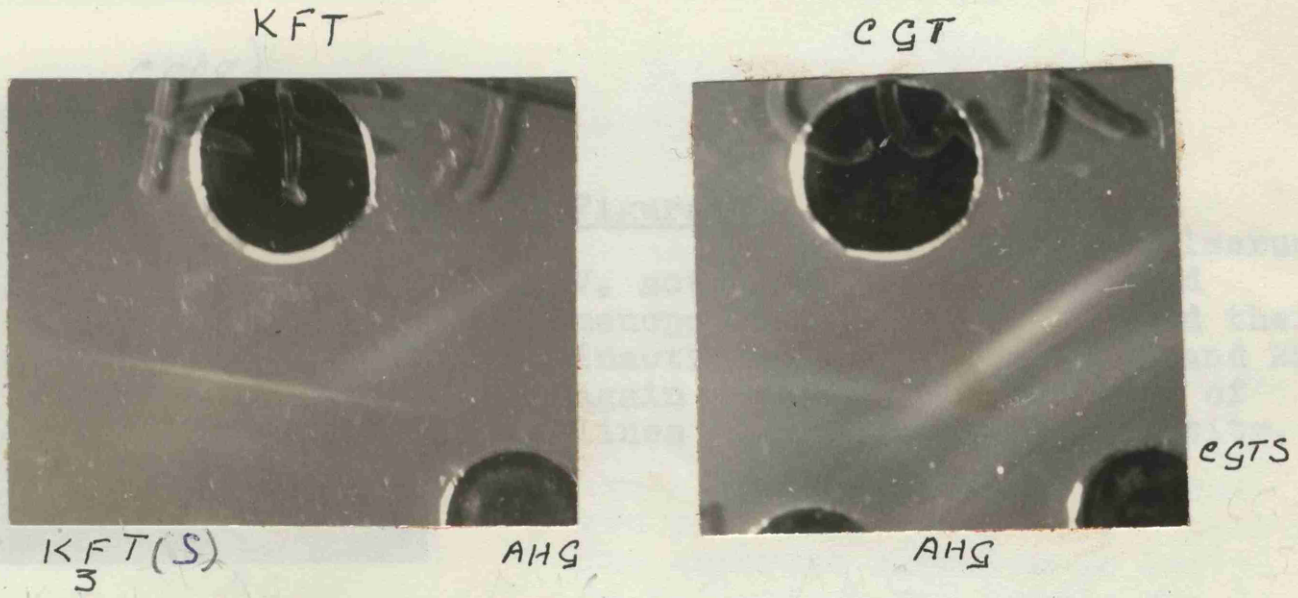


Figure 15 a and 15b.

These show the reaction of tannic extracts of Klinefelter and Pregnancy urines (KFT and CGT) against their own antisera (KFTS and CGTS) and also against anti-human globulin.
Note that a faint reaction for globulin is obtained but this is not identical with the lines shown by the antisera.

Figure 16.

Klinefelter Antisera V. (1) active extracts, Klinefelter (KFT), menopausal (HMG); (2) a weak extract, normal male (No♂) and (3) an inactive extract, hypoxed male (342).
Note multiple lines and identity.

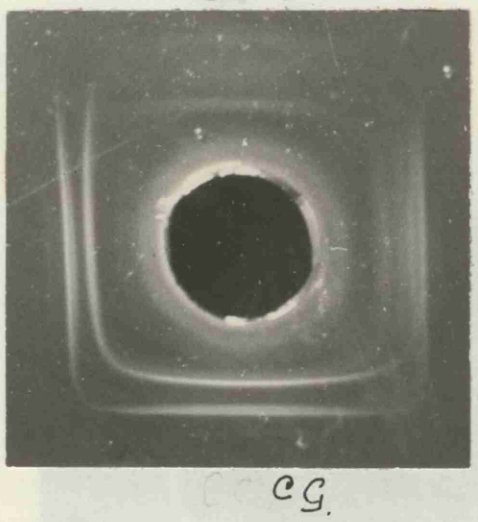


Figure 17.

Klinefelter antiserum V. active pregnancy (CG) and menopausal (MG) extracts and their inactive counterparts (CG8 and MG8). Again note the multiplicity of lines and reactions of identity.

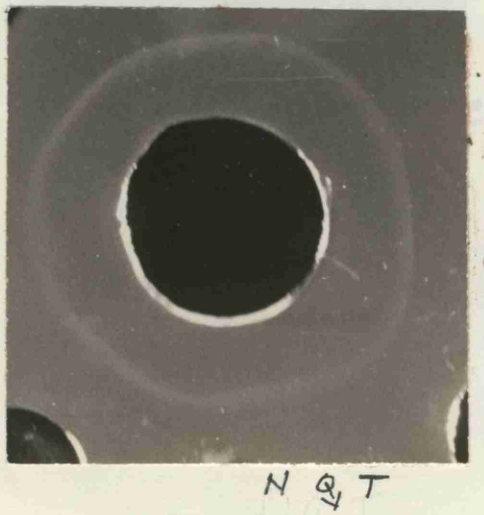


Figure 18.

Klinefelter antiserum, absorbed with child extract V. Kaolin and tannic extracts of Klinefelter (KFK, KFT), pregnancy (CGK, CGT) normal female (N♀ K, N♀ T) and normal male (N♂ K, N♂ T) urines.

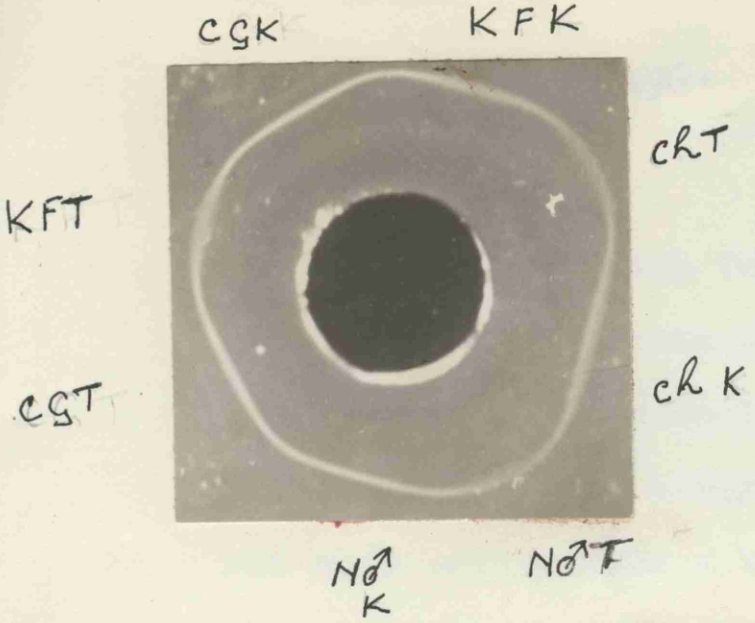


Figure 19.

Pregnancy antiserum absorbed with child extract V. Kaolin and tannic extracts of Klinefelter (KFK, KFT), pregnancy (CGK, CGT). Normal male(No♂K No♂T) and child's (ChK, ChT) urines.

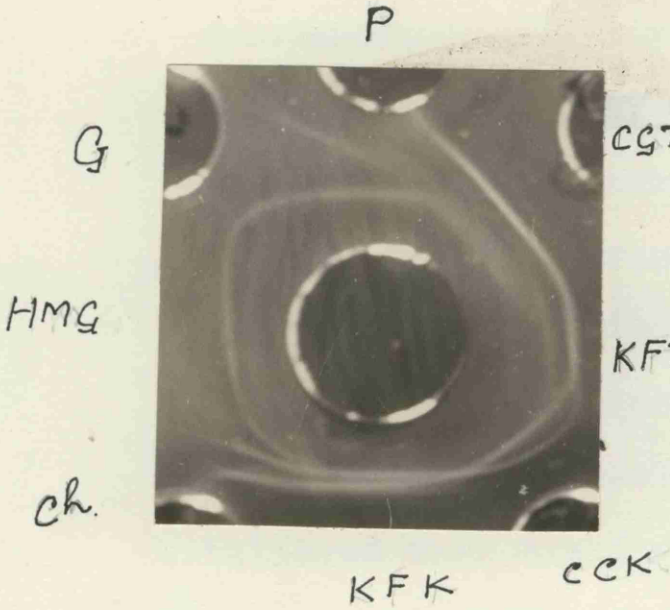


Figure 20.

Pregnancy antiserum V. Pregnyl (P), Gestyl (G), menopausal (HMG), child's (Ch), two Klinefelter (KFK, KFT) and two pregnancy (CGK, CGT) extracts. The apparent reaction to Gestyl is fallacious and merely due to union of lines from Pregnyl and Menopausal extract.

COLORIMETRY OF GLYCOPROTEINS.

Chemical Assay of Glycoproteins of the Urinary Extracts.

Introduction.

The serological tests have shown that the hormone extracts, even when purified contain antigens other than the hormone itself. Gel diffusion experiments showed several antigen-antibody systems, but such pattern could be obtained whether there was one chemical substance partly related and partly non-related to the hormone, or whether there were several substances chemically and immunologically allied to the hormone substance.

Tissue localisation experiments (Cruickshank & Currie 1958) have suggested that the non-hormonal antigens must be biochemically very similar to the hormone antigen, since they have always produced irreducible non specific reactions. Since it is believed that the gonadotrophins are glycoproteins or mucoproteins, it is only reasonable that we should try to gain more information of the chemical nature of the bulk of the non-hormone antigens, by chemical estimation of the glycoproteins in the hormone extracts.

If the intensity of the chemical reaction and its pattern of variation agree with the intensity and pattern of the serological reactions, this is strong evidence that all the antigens, hormonal or non-hormonal, are closely related glycoproteins.

Moreover, since biologically inert extracts (children's extracts), give serological reactions identical with reactions of the hormone extracts, it is essential to find out if they contain chemically detectable glycoproteins or some other chemical substance with identical antigenic behaviour.

The gonadotrophic hormones are considered to be glycoproteins since carbohydrates and proteins have been demonstrated in the "so called" pure extracts of the hormones obtained from different sources (Fevold et al 1937, Li et al, 1949, Morris 1955, Loraine 1958). Stran and Jones (1954) and Crooke et al (1954) have attempted to estimate gonadotrophins by testing urinary

extracts for proteins and sugars. There is at present no evidence that there is close correlation between the biological activity and chemical measurements such as ninhydrin colour, orcinol colour and ultra violet absorption (Loraine 1958, Martin & Emmens, 1961).

Anderson and McLaggan (1954, 1955) reported favourably on the use of acid diphenylamine reagent for the quantitative estimation of mucoproteins in benzoic extracts of urines. This reagent had been used for the estimation of fructose (Jolles 1910), inulin (Corcoran & Pope 1939, Alving, Rubin & Miller 1939), a serum constituent thought to be mucoprotein (Niazi & State 1948, Ayala, Moore & Hess 1951), and for mucoprotein foetin from foetal calf serum (Deutsch 1954).

The diphenylamine reagent (D.P.A.) was used by us to test for the glycoprotein content of urinary hormone extracts, according to the method of Anderson and McLaggan (1955).

A purple colour was obtained from such reactions, the intensity of which was measured against a control in a colorimeter (EEL).

Sometimes a greenish colour developed instead of a purple one and that was due to contamination of the solutions with some nitrates. The test was then repeated using fresh solutions.

A reading over 100 was discarded and a higher dilution of the extract solution was retested.

Technique.

A convenient volume (2.5 ml.) was pipetted into a special reaction tube. The diphenylamine reagent (D.P.A.) was then added in equal volume. The tube was stoppered with a perforated glass stopper and heated at 100°C. in a boiling water bath for exactly thirty minutes. It was then allowed to cool and the colour read in a photo-electric colorimeter (EEL). A blank of 2.5 ml. of the solvent of the hormone extracts was similarly

Table 1.

Accuracy and sensitivity of D.P.A. reagent in the quantitation of hormone extracts.

Dilution of extract.	Gestyl			Pregnyl			Kaolin KF extract		Tannic KF extract		Tannic Child extract	
	EEL	I.U.	M.U.U.	EEL	I.U.	M.U.U.	EEL	M.U.U.	EEL	M.U.U.	EEL	M.U.U.
Neat	70	2500	2560	50	4500	1280	71	40	51	40	25	0
1/2	42	1250	1280	26	2250	640	445	20	24	20	12	0
1/3	28	833	850	16	1500	427	32	15	15	13.3	8	0
1/4	21	625	640	13	1125	320	22.5	10	12	10	5.5	0
1/6	16	417	425	9	750	213.5	17	7.5	7.5	6.6	3	0
1/8	12	312	320	6	562.5	160	12	5	5.5	5	1.5	0
1/16	7	156	160	3.5	281.2	80	7	0	3	0	0	0

treated and noted as a zero control. A spectrum green filter (500-540 mμ.) was used for reading.

The test was performed for the following purposes:-

1. It was first performed on serial dilutions of Oestyl and Progyl to assess the ability of the D.E.A. reagent to produce a proportional increase in the colour intensity.(Table I, Figures 1 and 2).

2. The same experiment was then repeated using serial dilutions of crude and purified hormone extracts. (Table I, Figures 2 and 3.).

From these two experiments, three curves were constructed.

3. The test was then used to correlate the intensity of the colour reactions, with the mouse-uterus activity of 85 crude extracts, and 24 purified preparations. The results were plotted on the corresponding curves.

4. Finally, the test was performed to compare the intensity and variation of the colorimetric and serological reactions of several extracts.

Table 2.

D.P.A. Colorimetry of crude and purified extracts.

Antigen. 1/4	Kaolin EEL	Kaolin M.U.U.	Tannic EEL	Tannic M.U.U.
KF I	40	40	29	40
KF II	74	80	61	40-80
KF III	66	80	42	80
KF IV	52	20	39	20
KF V	80	40-80	69	80
KF 5	56	10		
KF 5	30	5		
CG I	92	over 160	75.5	160
CG II	95	160	83	160
CG III	62	160	57.5	80
CG IV	82	over 160	64	80
CG VI	57	80	48	80
CG 8	45	0		
N♀ I	31	10-20	52	10
N♀ 2	66	20-40	25	20
N♀ 3	32	20	34	20
N♂ 1	37	20	22	20
N♂ 2	20	5	16.5	10
N♂ 3	42	20	40	10
HMG 1	53	80	52	80
HMG 2	79	160	64	80
Child 1	65	0	40	0
Child II	25	0	21	0
N ♀ (Am)	72	10-20	74	20
Hyperpituitary	66	5	59	5
MG 8	38	0	2	0

KF = Klinefelter Urine.
CG = Pregnancy Urine.
CG8 = Pregnancy urine extracted at pH 8.
HMG = Human Menopausal Extract.
MG8 = Menopausal Gonadotrophin extracted at pH 8.
N ♀ (Am) = Normal female with amenorrhea.

Results.

Reference to Table I and to Figures 1 and 2 show that a straight line was obtained when serial dilutions of any given extract, crude or purified, were tested with the D.P.A. reagent. Table 2 also shows that the purified extracts as a whole produced lower colour intensities than the corresponding dilutions of crude extracts. Relative to the biological activity, Pregnyl and Gestyl produced less colour than tannic acid preparations, while the latter produced less colour than Kaolin preparations.

When colorimetric readings of 85 Kaolin extracts were applied to the corresponding colour/m.u.u. curve (Figure 2a), the colour reading was too high in 33 points, of which 22 points belonged to extracts of low activity (normal male, normal female, hypopituitarism, children's extracts). In twelve points, eight belonged to extracts of high m.u.u. activity, the colour intensity

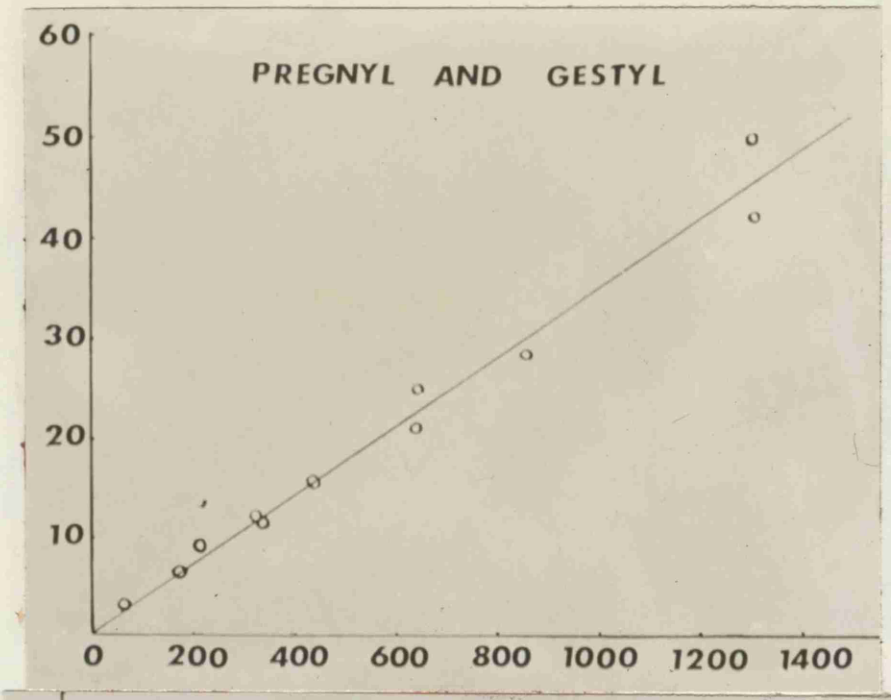
was lower than it should be.

In the case of the 21 tannic acid preparations tested, five produced more colour and two produced less colour than would be expected from their m.u.u. activities. (Figure 2b).

Figure 3 shows that the range and pattern of disagreement between colorimetry reactions and m.u.u. activities were the same as those for the serological tests, especially the precipitin and complement fixation reactions. Such disagreement was more marked if the colour intensity read below 25 or above 70-80 EEL. A dilution of $\frac{1}{4}$ of most extracts gave a reading within that range, but very occasionally $\frac{1}{2}$ or $\frac{1}{8}$ dilutions had to be used.

In the case of Pregnyl and Gestyl, there was no discrepancy between the colour intensity and the mouse uterus activity.

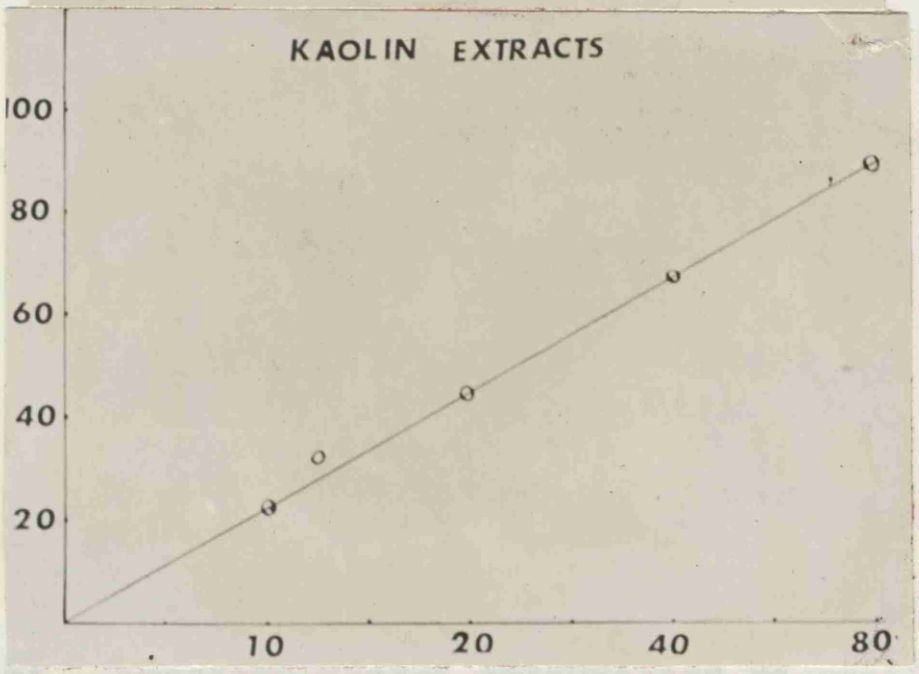
Reference to Table 3 shows that when the fractions obtained in Sephadex gel filtration were tested colorimetrically for



EEL reading

A

I.U. activity.

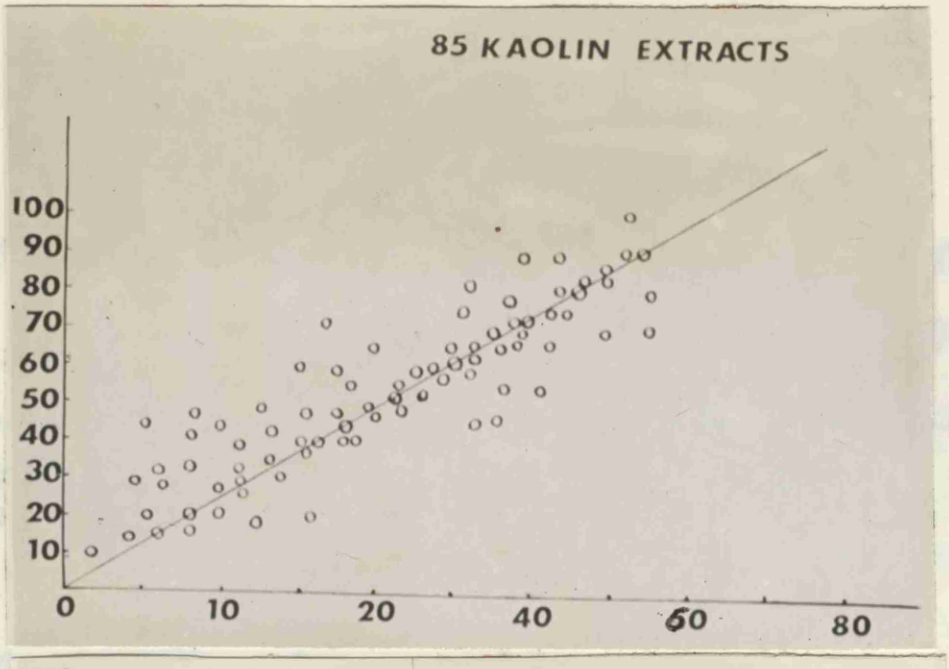


EEL reading

B

m.u.u. activity.

Figure 1. Colorimeter and biological activity. Serial dilutions of a) Pregnyl and Gestyl and b) Klinefelter Kaolin extracts. Colour reaction plotted against activity. Note straight line in each case.



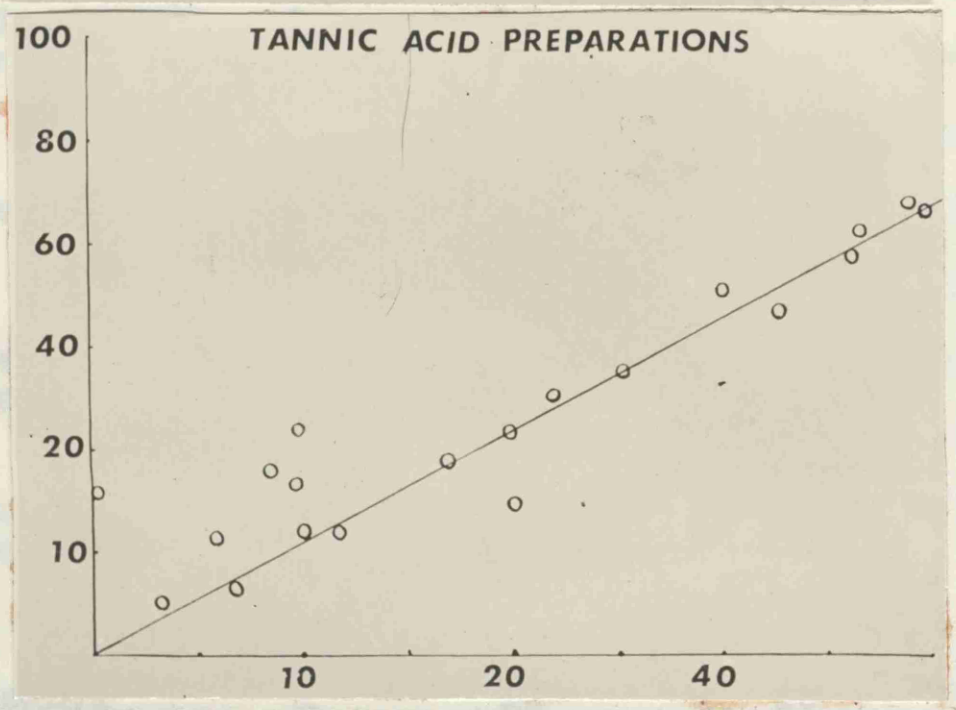


Figure 2. Plotting colour intensities of different preparations against their M.U.U.

A. 85 Kaolin extracts.
B. 21 tannic acid extracts.

Note that for the same M.U.U. tannic preparations give less colour than kaolin extracts. More points lie off the line with kaolin extracts.

Table 3.

Colorimetry of Sephadex Fractions.

Extract	Fraction No.	EEL Reading.	Activity of fraction (m.u.u.)	Activity of the original solution. (m.u.u.)
Klinefelter (Kaolin)	1	28	10	20
	2	6.5	5	
	3	-	-	
	4	-	-	
	5	-	-	
Klinefelter (Tannic)	1	18	5-10	20
	2	5	5	
	3	-	-	
	4	-	-	
	5	-	-	
Pregnancy	1	27.6	5	20-40
	2	10	10-20	
	3	-	-	
	4	-	-	
Pregnancy (Tannic)	1	17.8	5	20
	2	10	10	
	3	-	-	
	4	-	-	
Menopausal (Tannic)	1	19.5	10	10
	2	7	-	
	3	-	-	
	4	-	-	
	5	-	-	
Pregnyl	1	12	20	80
	2	3	40	
	3	-	-	
	4	-	-	
Gestyl	1	16	80	160
	2	4	20	
	3	-	-	
	4	-	-	

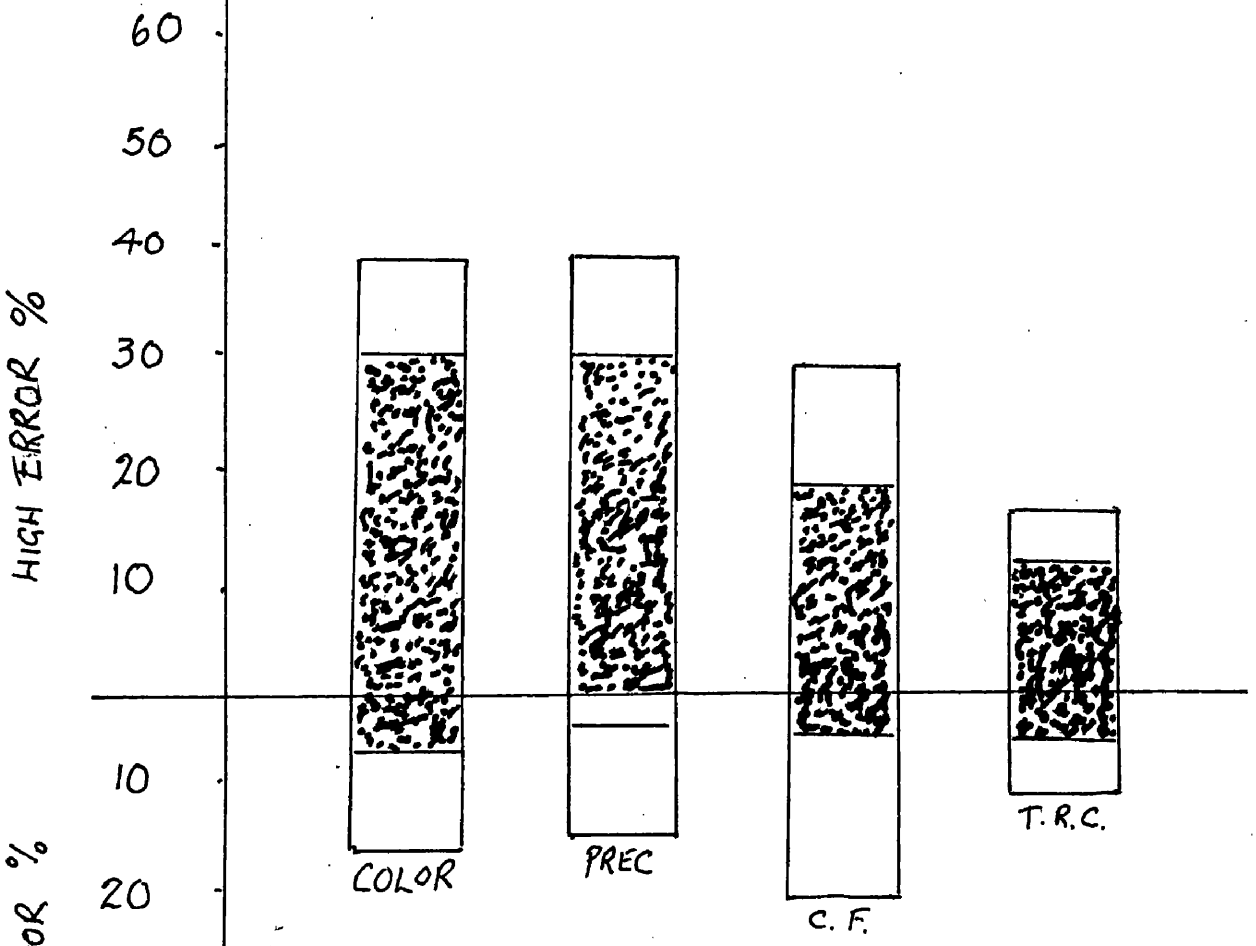
glycoproteins, the first fraction always gave a colour intensity between 10 and 20 EEL. The second fractions always gave a weaker reaction (5-10). No other fractions produced any colour with the DPA reagent.

Although the second fractions of the tannic acid pregnancy urine extracts and of Pregnyl preparations were very active in mice, their colour reactions were weaker than those of the corresponding first fractions. It seems very significant that only one line was produced in gel diffusion by postmenopausal extracts and only one Sephadex fraction of such extracts gave colour reactions with DPA.

The first fraction of Klinefelter extracts possessed the higher activity, the stronger colour reaction and the only component able to give gel diffusion reactions. Therefore, there seems to be a difference between chorionic and pituitary gonadotrophins, detected by Sephadex. Moreover, it seems that Sephadex can isolate fractions with

Figure- 3-

Percentage discrepancy between m.u.u. and different hormone assay tests (using Kaolin extracts).



High error percentage = proportion of extracts giving colour intensity higher than expected from their m.u.u.
Low error percentage = proportion of extracts giving colour intensity lower than expected from their m.u.u.
Color. = colorimetry results. Prec.= precipitin results. C.F.= complement fixation results.
TRC = haemoagglutination results.
Shaded areas indicate the proportion of extracts of low m.u.u. giving rise to error.
Non-shaded areas indicate the proportion of extracts of high m.u.u. activity giving rise to error.

biological activity, and such a small content of glycoproteins that they do not give any precipitin reactions.

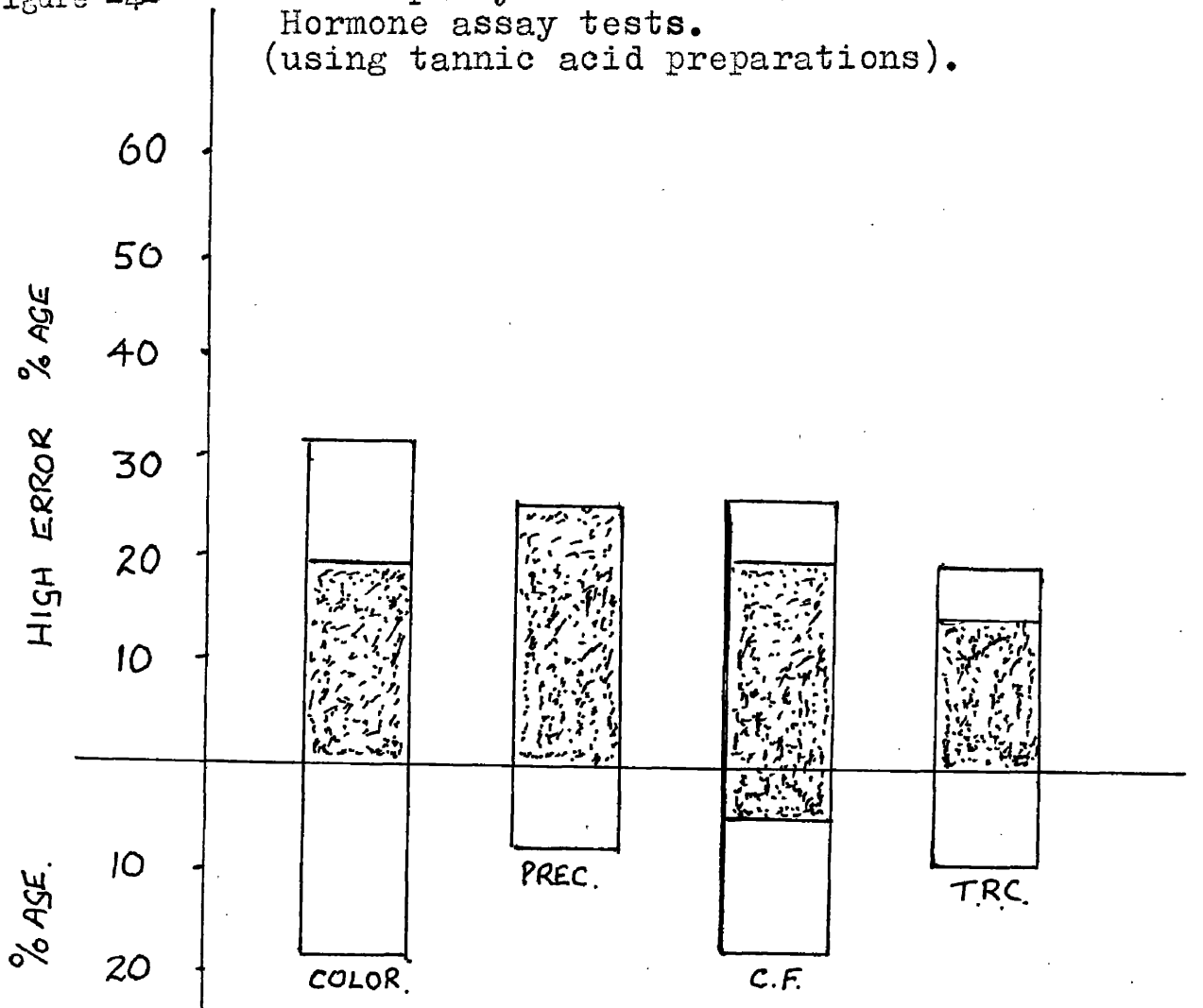
Comparison of the mouse uterine units activity and the colorimetric results of the highly purified benzoic extracts Gestyl and Pregnyl with the same estimations in cruder extracts indicates that as purification occurs the amount of glycoprotein diminishes.

A further comparison of all the tests in relation to biological activity is shown in Figures 3 and 4. The first illustrates the degree of discrepancy between the test and the biological activity in tests carried out with Kaolin extracts. In the second, the results for tannic extracts are shown.

In both instances it can be seen that the discrepancy was least in the haemagglutination tests. The greatest discrepancy was shown by the colorimetric and precipitin tests both in extracts of high and low biological

Figure -4-

Discrepancy between m.u.u. and other Hormone assay tests.
(using tannic acid preparations).



High error percentage = proportion of extracts giving colour intensity higher than expected from their m.u.u.
Low error percentage = proportion of extracts giving colour intensity lower than expected from their m.u.u.
Color. = colorimetry results. Prec.= precipitin results. C.F. = complement fixation results. TRC = haemoagglutination results.
Shaded areas indicate the proportion of extracts of low m.u.u. giving rise to error.
Non-shaded areas indicate the proportion of extracts of high m.u.u. activity giving rise to error.

activity. Complement fixation occupied an intermediate position but the discrepancy was still much greater than with haemagglutinins. Tannic acid purification altered the quality of the discrepancy rather than the degree.

Summary:

1. The D.P.A. reagent successfully produced colour with all the urinary extracts as well as with Gestyl (P.M.S.). The colour intensity varied directly with the dilution used up to a reading of 100 in the colorimeter.
2. Cruder extracts produced stronger colour than purified ones.
3. The colour intensities were used to plot a curve for the biological activity of crude and purified extracts. The discrepancy was least with readings between 20 and 70.
4. The discrepancy between the m.u.u. activities and colour intensities was mainly due to low activity or inert extracts giving too strong colours. This corresponded with

discrepancies between the m.u.u. and the precipitation and complement fixation reactions in general.

Conclusions.

1. D.P.A. is an efficient reagent for the quantitation of glycoproteins in hormone extracts.

2. D.P.A. is successful in assaying the biological activity of highly active extracts. However, with low activity or inert extracts there is considerable discrepancy.

This is of the same order and magnitude as that of the serological reactions.

It is therefore reasonable to conclude that all these reactions actually measure the same component or components of the extracts.

EXPERIMENTS ON FRESH URINE.

Experiments on Fresh Urines.

Fresh urine was obtained from 32 pregnant women in the first five months of their pregnancy, from 16 normal females, mostly in the first half of their cycles, from 10 normal males, 5 Klinefelter patients and 3 post-menopausal women. Two specimens of children's urine were obtained, one of them containing considerable proteinuria. Of the 32 pregnancy urine samples, one showed heavy proteinuria, and another one was obtained from a patient with a hydatiform mole pregnancy.

Each urine sample was mixed with a little Celite powder, and then centrifuged until it was quite clear. The sample was diluted with distilled water to a final dilution of 1 in 5, which resulted in an isotonic solution (McKean 1960). Normal saline was then used to make further dilutions of 1/10, 1/20, 1/40, and 1/80.

Using antiserum to pregnancy urine extracts (CGT antiserum).

Precipitin Reactions:

Eight of the 32 pregnancy urines gave no reaction at dilutions higher than 1/10 while the rest reacted up to dilution of 1/80. All the other urines reacted with the antiserum to pregnancy extracts, except the children's urine not containing proteins. Most of the urines of normal males and females reacted to a dilution of 1/10, but three normal female urines and four normal male urines reacted to dilutions of 1/20 to 1/40.

Three of the Klinefelter urines reacted to dilution 1/80, the other two to dilution 1/20. Two IHG urines reacted at dilution 1/40, the third at dilution 1/10. The pathological children's urine reacted to dilution of 1/10.

All these urines were also tested against the same antiserum by the immunocrit method.

The amount of precipitates produced coincided very closely with the results of the dilution technique.

Complement fixation reactions:

The 1/5 dilution of urine was very rarely anticomplementary, so it was chosen as the fixed dilution of the antigens. Quantitation was done by varying the amount of MHDs of complement per tube. The antiserum was also used in a constant dilution (1/4-1/8).

All the urines tested gave a positive reaction, except the normal children's urine. Five of the pregnancy urines reacted with less than 4 MHDs, the rest reacted with 7 to 10 MHDs. All normal female urines reacted with 2 to 4 MHD except one which reacted with 6 MHDs. Four male urines reacted with 5-6 MHDs., while the rest reacted with less than 5 MHDs. Three Klinefelter urines reacted with more than 7 MHDs., one with 5 MHDS and one with 3 MHDs. One MHG urine reacted with 10 MHDs, the other two with 2 and 3 MHDs.

The pathological children's urine reacted with 3 MHD.

Haemoagglutination inhibition reactions:

All the urines gave positive reactions except both children's urines, which gave no reaction. The pregnancy urines reacted at a dilution of 1/80 except three of them, which reacted at dilutions 1/20 and 1/40. Normal male and female urines reacted at dilutions of 1/20 or less, except two male and two female urines, which reacted at dilutions of 1/40 and 1/80. One HMG and three Klinefelter urines reacted at dilution 1/80, while the rest reacted up to dilutions of 1/20 to 1/40.

These results indicate that antisera to HCG are not, strictly speaking, specific for chorionic gonadotrophins, no matter which serological reaction is used. However, the quantitative reactions seem to indicate a difference in the extent of reaction which could serve as basis for a practical test of pregnancy. In such case, the precipitin

reactions show about 20% false positives and about 11% false negatives. The complement fixation reactions show 14% false positives and 7% false negatives, while the haemoagglutination reactions show 11% false positives and 4% false negatives.

Using antiserum to tannic extracts of Klinefelter urine:

Precipitin reactions:

Four pregnancy urines and two Klinefelter's urine reacted at dilutions of 1/10 and 1/20, while the rest of the pregnancy and Klinefelter urines reacted at dilutions of 1/40 and 1/80. Five normal female and five male urines reacted at dilutions 1/40 - 1/80, while the rest did not react at dilutions above 1/20. One HMG urine gave reactions only up to dilution 1/10, while the other two reacted up to 1/40 and 1/80. The pathological children's urine reacted at a dilution of 1/20, while the normal urine reacted only at 1/5 dilution.

Complement fixation reactions:

All the urines produced positive reactions. Six of the pregnancy urines and one of the Klinefelter's urines reacted with less than 5 MHDs, while the rest of the pregnancy and Klinefelter urines reacted with 5 or more MHDs., usually with 7 to 10 MHDs. Most of the normal male urines reacted with 4 - 5 MHDs, while most of the female urines reacted with 2 - 3 MHDs. Four female urines and five male urines reacted with 5 to 8 MHDs. One postmenopausal urine reacted with 3 MHDs, the other two with 6 MHDs. The pathological children's urine reacted with 4 MHDs, while the normal specimen reacted with 3 MHDs.

Haemoagglutination inhibition reactions:

All the urines produced positive reactions except the normal children's urine. Three pregnancy urines, one Klinefelter's urine, and one HMG urine reacted at dilutions 1/10 to 1/20, and not more. The other

pregnancy, Klinefelter and postmenopausal urines produced positive reactions up to dilutions 1/40 and 1/80. Two female urines and three male urines reacted at dilutions 1/40 or more, while the rest reacted up to dilutions of 1/20. The pathological children's urine reacted at a dilution of 1/20.

These results indicate that antisera to pituitary hormones are not really strictly specific for pituitary hormones. As a matter of fact they react equally strongly with chorionic gonadotrophins. Therefore, such antisera cannot be used to differentiate between those two types of hormones, but they may be of some use in assaying the total gonadotrophic activity in urines. In such application, the precipitin reactions produced falsely high results in 16% and unduly low results in 10%. The complement fixation reactions produced too high reactions in 16% and too low reactions in 11%. Finally, the haemoagglutination inhibition reaction produced

unduly high results in 9% and unduly low results in 7% of urines tested.

It therefore seems obvious that antisera to pregnancy extracts are by no means more specific than antisera to pituitary hormones. (Wide & Gemzell 1962), nor vice versa (Butt et al 1961). Using diluted urines in the haemoagglutination inhibition test, seems to offer a good possibility of successfully assaying the total gonadotrophic activities in all types of urines, rather than differentiating between them. However, since normal female urines produced low incidence of error with the antiserum to pregnancy extracts, the practical value of haemoagglutination for the diagnosis of pregnancy seems clear.

DISCUSSION.

Discussion.

The immunological reactions reported above may be considered from several points of view. A number of academic questions suggest themselves, such as the nature of these reactions, and their relationship one to another. The problem of species specificity must be considered. There is also the question of whether the reactions have any degree of specificity for gonadotrophic hormone per se, and whether it is possible to differentiate hormones arising from different organs in the same species. It may be possible to deduce something as to the nature of the hormone and its relationship to the antigens involved in these reactions. In addition there is the practical problem of the possible use of these tests for the estimation of hormone activity.

Precipitins.

With regard to the precipitin tests probably the most important fact is that, in our hands, these were species specific. No

cross precipitin reactions could be obtained between Gestyl, a proprietary preparation of pregnant mare's serum and antisera to human gonadotrophin extracts, nor were there any between these extracts and the antiserum to Gestyl. Chow et al (1942) and Henry & Van Dyke (1958) working with a preparation of interstitial cell stimulating hormone obtained similar results. Cruickshank & Currie (1958) however were inclined to think that the precipitin reactions of chorionic gonadotrophin were not species specific. Witebsky et al, (1955). agreed with the latter. This species specificity immediately raises a doubt regarding the relationship between precipitins and hormone activity. Nevertheless the precipitates obtained from the tests when injected into animals showed some evidence of biological activity indicating that at least some hormone is involved in these reactions. This was also a finding of Henry and Van Dyke (1958).

No evidence of organ specificity was

apparent. Although the highest titres were obtained with the most active extracts, it was impossible to differentiate between these extracts. Cross precipitation was the rule. McKean (1960) has claimed that it was possible to differentiate one type of gonadotrophin from another but Cruickshank & Currie (1958), Butt et al (1960-61) and Rao and Shahani (1961) found that precipitins were not organ specific.

Apart altogether from differentiating one type of gonadotrophin from another, the titres of reaction obtained when testing several preparations of the same type of gonadotrophin were very variable and bore practically no relation to the biological activity of these extracts.

With a specific antibody reaction the purification of the antiserum or the antigen should make no difference to the strength of reaction. In the case of these precipitin reactions purification of the antigens

reduced the titres of reaction considerably. From this and the fact that inert and low activity extracts produced fairly strong precipitation with the antisera it was obvious that multiple antigens were involved in these precipitin reactions.

The same results were obtained whether the precipitin reaction was carried out by the inter-facial ring dilution technique or by the immunocrit method. In view of the obvious multiplicity of the antigens we used the Ouchterlony gel technique to try and differentiate qualitatively between the antigens and if possible the various types of gonadotrophin.

Preliminary experimentation showed that technique and time of reading the results played an important part in interpretation. It was obvious from the first that a number of antigens and therefore antibodies took part in the precipitin reactions. This was true for all extracts and antisera used. Cruickshank

& Currie (1958) in an exhaustive study of gel diffusion studies of gonadotrophins obtained similar results. Chorionic gonadotrophins and extracts of normal male urines produced two lines, while menopausal extracts gave only one line. Butt et al (1960, 1961) observed two lines with chorionic gonadotrophin and also with menopausal gonadotrophin but only one line with gonadotrophins of pituitary origin. Brody & Carlstrom (1961ab)were able to demonstrate two lines with extracts of urine from both pregnant and non-pregnant women. In our series of experiments using antigens and specific antisera the number of lines tended to vary with the individual serum. In general however, Klinefelter extracts produced three strong lines, pregnancy extracts produced two strong lines and menopausal extracts one strong line. Weaker lines were produced in all cases.

Purification of the antigens by tannic acid precipitation, ion exchange resins, or

adsorption with tricalcium phosphate made no difference to the number and distribution of lines we obtained. Further purification by ammonium sulphate and Sephadex gel reduced the number of lines but at least two lines remained and in the case of the ammonium sulphate separation two lines were shown by several fractions. Similarly, antisera to purified extracts made no difference to the reactions and even using the γ-globulins of antisera still produced the same multiplicity of lines, equally without avail.

These reactions were certainly not organ specific. Cross reactions occurred with all preparations and their antisera and as can be seen from the illustrations these lines showed evidence of identity.

Similar cross reactions were obtained by Cruickshank and Currie (1958). Their anti-menopausal serum reacted with purified chorionic gonadotrophin, extracts of male urine and even thyroid stimulating hormone.

Henry and Van Dyke (1958) using antiserum to sheep interstitial cell stimulating hormone obtained cross reactions with ox gonadotrophins. Butt et al (1960, 1961) observed two lines in gel diffusion when anti-menopausal serum reacted with either menopausal or chorionic gonadotrophins.

According to our results the reactions were species specific in that Gestyl failed to give lines with any of the antisera. As noted above, however, Henry & Van Dyke (1958) obtained cross precipitation between antisera to sheep gonadotrophins and ox gonadotrophins although not with hog or human chorionic gonadotrophins, so that the species specificity may not be so strict as might seem.

As with the ordinary crude interfacial ring technique there seemed to be some relationship between these reactions and biological activity. This was most clearly seen in experiments with fractions separated by the ammonium sulphate and Sephadex techniques. Reactions were only obtained with those

fractions which possessed activity. It is to be noted however that these fractions produced more than one line in gel diffusion, indicating that even these techniques were unable to do more than separate the antigens into several groups. It occurred to us that the extracts might contain some serum globulins and tests were made for their presence. The results showed that globulins were indeed present in traces but the lines produced in gel diffusion showed no evidence of identity with those obtained in the reaction between the extracts and their antisera.

Despite the fact that lines were only obtained with active fractions separated by the techniques mentioned above and these lines were identical with others shown by the mother extracts, it has to be admitted that this is no proof of hormone identity. Identical lines were obtained with low activity and inert extracts. Rao and Shahani (1961) also obtained strong reactions with

inert extracts. Several interpretations are possible. One interpretation involves a consideration of the nature of the antigens and their relationship to gonadotrophic activity and will be considered later. Other two are as follows. Either the reactions have nothing to do with hormone activity or the separation of the antigens is not complete enough. It would seem a remarkable coincidence that only those fractions possessing biological activity should produce lines if the reactions have no relationship thereto. At the same time it is apparent from the multiplicity of lines that separation of the antigens is indeed far from complete.

In an attempt to solve this problem absorption experiments were carried out. Excess antigen in the form of inactive child's extract was added to antiserum and the precipitate filtered off. The supernatant was then tested against the various extracts. A single line was produced in

each case. The intensity of this line appeared to be the same whether the extract was of high or low activity. The line was identical in each case. This might seem to be a successful reduction of the reaction to the hormonal fraction. In another series of experiments, however, it was found that the absorbed antiserum still reacted with inert extracts, even child's extract which had been used for absorption. The line produced by the child's extract was identical with that produced by the other extracts. This would indicate that during absorption the weak antibodies had been successfully absorbed but that a strong antibody had been incompletely taken up. In addition this antibody reacted with an antigen in an inert extract, namely from child's urine, and this antigen appeared to be identical with those in other extracts producing reaction. It is evident therefore that a single line in gel diffusion cannot be a reliable indicator of biological activity.

Several investigators have suggested that the antigenicity of these hormones is altered by the chemical processes involved in their extraction (Werner, 1938; Ostergaard, 1942). Brody & Carlstrom (1960, 1961a,b) using anti-chorionic serum obtained two identical lines with fresh sera from pregnant and non-pregnant women. By dilution a single line could be obtained from the serum of non-pregnant women. Using anti-chorionic and anti-Klinefelter sera, we obtained three lines from fresh sera of pregnant women, and three lines from sera of non-pregnant women, all being identical. Moreover, we obtained one line from fresh urine of each of pregnant women, non-pregnant women and normal males. This single line was identical in all urines, and also identical with one of the lines of the fresh sera of pregnant and non-pregnant women. Since the sera reacted with antisera to extracts it indicates that the antigens are of similar nature in both fresh serum and urinary extracts. The reaction produced by

fresh urine indicates the presence of an antigen common to urine and serum. The fact that only a single line is produced by urine would suggest that the other antigens are too dilute to produce a reaction.

Complement Fixation.

Use of the various antibody techniques to study the pattern of rise and fall of antibodies during immunisation of our animals made one suspect that the various antibodies were reacting with different antigens. Although complement fixation tests are generally considered to be more sensitive than precipitin tests it is noteworthy that precipitins appeared earlier than complement fixing antibodies but the latter eventually reached a higher titre. After cessation of immunisation on the other hand complement fixing antibodies lingered longer, than precipitins. These complement fixing antibodies, unlike precipitins, were not species specific since cross reactions were obtained

between the various hormone extracts and their antisera and Gestyl and its antiserum.

Brody & Carlstrom (1960) made claims regarding the organ specificity of complement fixation tests. We could demonstrate no such specificity. Cross reactions occurred with all antisera and antigens. In the case of individual antigens it was possible to demonstrate a higher titre with the specific antiserum but the range of titres with various antigens of a similar nature made it obvious that it was impossible to designate an unknown antigen.

There has been much controversy regarding the relationship of these reactions to biological activity. It seemed to us that they were related in some degree to hormone activity. The strongest reactions were usually obtained with the most active hormone extracts. This is in agreement with Twombly (1936), Rowland and Parkes (1939), Got et al (1959), McLean (1960) and Brody &

Carlstrom (1961, 1962). A directly opposite opinion is held by Van den Ende (1939) and Rao & Shahani (1961), while Henry and van Dyke (1958) and Butt et al (1960, 1961) are uncertain. A considerable loss of antigenicity was noted in our experiments whenever an extract was purified.

Brody & Carlstrom (1961) are probably the strongest protagonists of the view that complement fixation is hormone specific. This despite the fact that, using a higher purified preparation of chorionic gonadotrophin containing 6000 International units/mg., they obtained strong complement fixation with fresh serum from normal non-pregnant individuals. Rowland & Parkes (1939) and Rao & Shahani (1961) obtained positive complement fixation with inert and low activity extracts. Similar results were obtained in our experiments. Further, fresh urines and serum from non-pregnant subjects gave positive reactions in the hands of Eichbaum et al (1935, 1937) and Van den Ende (1939).

We found difficulty with fresh serum in these experiments because of apparent non-specific fixation of complement. Complement consumption tests with fresh urine from Klinefelter patients, pregnant individuals, normal males and females, normal children and children with albuminuria were carried out. Positive results were obtained in all cases with the exception of the urine from normal children. The reactions in a considerable proportion of the normal adult male specimens were almost as strong as in the average pregnancy case. Contrariwise 16 per cent of the pregnancy specimens produced a weaker reaction than the average male.

Nevertheless the results obtained with the extracts would tend to indicate that we are correct in our assumption that the reaction is partly hormone specific but, that apparently several antibodies and therefore several antigens are involved. It is obvious that this type of test could not be

used with any certitude of reliability as a test for the detection and assay of gonadotrophins. This opinion is strengthened by the observation that complement fixation tests carried out with the fractions of extracts separated on Sephadex columns were always strongest with Fraction 1, whereas biological activity was mostly concentrated in Fraction 2.

Haemagglutinins.

Using the haemagglutinin technique of Boyden (1951), Vide and Gemzell (1960) obtained positive reactions with highly purified extracts of pregnancy urine (Pregnyl) as antigen with a specific antiserum. In their immunising programme they used Ramon's adjuvant together with Pregnyl over a period of 5 to 6 weeks. At the end of this time the haemagglutinin titre was 1 in 6400. With our technique of injections we frequently obtained a titre of 1 in 10,000. The use of Ramon's adjuvant increased the titre slightly. This immediately differentiates

this test from others in vitro reactions such as precipitin and complement fixing antibodies which were not increased by adjuvant.

Wide & Gemzell (1960) also made use of the haemagglutinin inhibition technique of Read (1958) and Stone and Read (1958) and found this even more satisfactory than the ordinary test. They claimed that both reactions were very specific for both gonadotrophins and for the organ of origin. They obtained no cross reactions with human menopausal or other pituitary gonadotrophin, or growth hormone. As a result they claimed that the reaction was specific for pregnancy.

Butt et al (1960, 1961) and Rao and Shahani (1961) doubted the organ specificity of these antibodies. We obtained strong cross reactions between pregnancy and potent non-pregnancy extracts and their antisera. The more active extracts produced the stronger reactions. In addition each type of hormone seemed to react more strongly with its own

antiserum. Within each group however there was only rough correlation between the titres and the different biological potencies shown by the individual extracts. Both organ specificity and hormone specificity appear to be still open to question. We obtained positive reactions with inert extracts but the discrepancy between the biological activity and the in vitro tests was certainly not so marked as with precipitins and complement fixing antibodies. In addition purification of antigens made little or no difference to the titre of haemagglutinins. On the other hand Rao and Shahani (1961) found that hormone extracts made inert by boiling gave strong positive haemagglutination.

From the immediately practical point of view it seems possible to use this technique as a laboratory test for pregnancy on specimens of urine. The titre of reaction was sufficiently high to differentiate pregnancy specimens from most other specimens from non-pregnant females. As in all

pregnancy tests, however, it is probably of little use when dealing with patients approaching the menopause. This again indicates that these antibodies are not organ specific. The explanation why this test can possibly be applied successfully to urine as a test for pregnancy is to be found in our gel diffusion studies with fresh urine. There it was shown that while urinary extracts contained multiple antigens identical to those in serum, fresh urine only appeared to contain one. It is obvious that many antigens are too dilute in urine to interfere with the haemagglutinin test.

Neutralisation.

Neutralisation of gonadotrophins was one of the first aspects of hormonal immunology studied. This stemmed from the observation that with prolonged treatment animals ceased to react to gonadotrophins.

Because of the repeatedly observed disagreement between in vitro reactions and

neutralisation it was thought for a time that neutralisation was not an immunological phenomenon (Twombly, 1936; Gegerson et al, 1936; Van den Ende, 1939). Twombly (1936), Gegerson et al (1936), Van den Ende (1939) and Cole et al (1957) have all found that complete precipitation of antihormone sera only slightly affects their neutralising power. This also we have found. Recently Butt et al (1960, 1961) reported results which suggest that the test animal is of prime importance. They produced antisera to human pituitary gonadotrophin and human chorionic gonadotrophin. It was found that antiserum to chorionic gonadotrophin inhibited the action of human pituitary gonadotrophin, human menopausal gonadotrophin and human chorionic gonadotrophin in both mice and rats. Antiserum to pituitary gonadotrophin inhibited the activity of all hormones in mice but failed to inhibit pituitary gonadotrophins in rats.

Most observers are now inclined to

belive that neutralisation is an antibody reaction although the exact mechanism remains obscure.

Brandt and Goldhammer (1936), Gustus et al (1935) and Rowlands (1937) believed that antihormones were species specific. Thompson and Cushing (1934) however found that antisera to pregnant mare's serum neutralised human, sheep and pig pituitary hormones. Collip (1937) reported that antiserum to human pituitary gonadotrophin inhibited the activity of pregnant mare's serum. There may be some variation from species to species however. Henry & Van Dyke produced an antiserum to sheep gonadotrophin and found that while it inhibited pig gonadotrophin it failed to react with human gonadotrophin.

In our experiments we found no evidence of species specificity. Antisera to human extracts inhibited the action of pregnant mare's serum and vice versa. Indeed, antisera to Gestyl proved to be the most potent

neutralising agents we produced.

The question of organ specificity has also resulted in controversy. Selye (1934) and Fluhman (1935ab) found that antisera to chorionic gonadotrophin was not effective against human pituitary extracts. Similarly Rowlands (1938) claimed that antisera to pregnant mare's serum were not effective against horse pituitary gonadotrophins. Fremery and Scheygrond (1937), Brandt and Goldhammer (1936) however, demonstrated cross neutralisation between human chorionic and pituitary gonadotrophins and their antisera.

The results of our own work indicated that antisera to chorionic and other urinary gonadotrophins were equally powerful in inhibiting both their corresponding hormones and others. Sometimes there appeared to be a slight degree of selectivity but this was negligible.

There seems little doubt that the neutralising power of these antisera is directly related to the presence of the

hormone in the original antigenic material used for immunisation. Antisera to low activity or inert extracts had no powers of neutralisation. This is in agreement with Fremory & Scheygrond(1937), Meyer & Wolfe (1939) and Cole et al (1957).

The mechanism of neutralisation is not clear. It has been suggested that it is really due to precipitation of the antigens by the antisera and may not be a specific action at all. This is only likely to be true if the hormone and antihormone were injected simultaneously at the same site. However, we found, as did Cole et al (1957) that injection of the hormone and antiserum at separate sites did not affect the ability of the antiserum to inhibit hormone activity. Also, since antisera to Oestyl possessed powerful cross-species neutralising power despite the fact that they possessed no cross precipitin antibodies, indicates that neutralisation is

not due to precipitation of the hormone. In relation to this point it is interesting to recall Eichbaum's (1937) study of the phases of antibody formation in relation to gonadotrophins. Precipitins were the first to appear, followed closely by complement fixing antibodies while neutralisation only appeared much later. The reverse was observed when immunisation was discontinued, precipitins and complement fixation disappearing while neutralisation was unimpaired. We noted similar results in our studies. In addition haemagglutinins were found to appear almost at the same time as neutralising antibodies and like them lingered after cessation of immunisation.

Correlation of the different serological reactions:

In our work, the species specificity seems to isolate precipitin reactions in a separate class from the other reactions. The pattern and rate of rise and fall of titres seem to indicate that different factors

govern different reactions given by the same antiserum (Meyer & Wolfe 1939, Gegerson et al 1936, Gustus et al 1935, Bachman et al, 1934). We have found that precipitin and complement fixation behave similarly in that they showed only rough correlation with biological activity. This agrees with Van den Ende (1939), Twombly (1936), and Got et al (1959). Neutralisation and haemoagglutination on the other hand did appear to have a closer relationship with the hormone activity. However, while antisera to inert extracts gave haemoagglutination reactions, they did not have neutralisation powers.

Complete absorption of antihormone sera leads to decrease in the precipitating and complement fixing powers of the supernatant, while the neutralising powers were hardly affected, indicating they were different reactions or may be different antibodies. This is in agreement with many other workers like Van den Ende (1939), Meyer & Wolfe et al (1939), Eichbaum/(1937), Collip (1934, 1935, a, b

1937), Thompson (1941), Butt et al (1961), Rao & Shahani (1961), who all believe that the in vivo and the in vitro reactions of antihormones sera are not related.

The power of antihormone sera to augment instead of to inhibit hormone preparations in experimental animals has long been reported.

In our experiments, this augmenting power seemed usually to precede the neutralising powers. At some stage the antiserum could possess both functions simultaneously, depending on the dilution used. These findings agree with those of Thompson (1937), Meyer & Wolfe (1939) and Cole et al (1957), who also found, as we did, that normal rabbit sera did not have this ability to augment. The two reactions seem to arise from the same process, the difference in effect being due to a change in the "nature" of the antibody molecule rather than its amount, since after discontinuing the immunization of rabbits, augmenting powers did

not reappear in their sera.

The γ-globulin fraction of antihormones sera does not seem to be the only part of the globulins responsible for the serological reactions. The α and β fractions seem to take a more constant role in precipitin than in neutralisation reactions, while γ globulin seers to account for most of the neutralisation and haemoagglutination reactions of antihormone sera.

Several authors have found that the "antihormones" are distributed between the fractions of the globulin proteins (Harrington & Rowlands 1937, Thompson 1937, 1941, Hartman et al 1940). Gegerson (1936) found that the globulins carried hormone specific and hormone non-specific antibodies.

These findings suggest that the serological reactions may not be intimately related. They may fall in two or more classes of reactions (Thompson 1941). There seem to be antibody reactions wholly related to the biological activity of the antiserum and antigen. These

(true antihormones) may include neutralising and haemoagglutination reactions. On the other hand some antibody reactions may be only partly or not at all related to the biological activity of the antiserum and antigen. This may include complement fixation and precipitation reactions. If this is true, then obviously one cannot expect the second class of reactions to be successfully applied for the detection and assay of gonadotrophic hormones, in extracts, biological fluids or tissues.

Also, if true, it means that the specificity of any reaction can be enhanced not only by purifying the antigen but also by separating the hormone specific from the hormone non-specific antibodies in the antihormone sera before using them.

The Nature of the Antigens.

There appear to be many antigens in gonadotrophic extracts of urines and despite all attempts at purification there is an irreducible number in each extract. This

opinion is held by many observers among them Werner et al,(1938), Thompson (1941) and Henry and Van Dyke (1958). Purification seems to effect quantitative rather than qualitative changes. It is to be noted also that with every purification there is a loss of total activity although the relative potency of the extract may increase.

Some of these antigens appeared to have no relation to the hormonal activity at all. This seemed to be obvious from the fact that low activity and inert extracts gave quite strong reactions with the antisera. This was observed even when antisera to relatively pure extracts were prepared.

Gel diffusion tests provided visual evidence of the multiplicity of antigens in extracts. They also showed that the same antigens were present in active and inert extracts. Further experiments with ammonium sulphate and Sephadex fractions of active preparations showed that only the active fractions produced lines of reaction.

Comparison with crude preparations, however, showed that the lines obtained, with the active fractions were identical with lines in both active and inert extracts. Two other points are to be noted at this time. Each fraction produced several lines of reaction indicating the presence of several antigens, and secondly biological activity is not confined to one fraction.

Attempts have been made to absorb out the antibodies to biologically inactive antigens by treating the antisera with inert extracts. This reduced the lines of reaction in gel diffusion to one. Despite this however, it was obvious that this single line had nothing to do with hormonal activity since it was found with active and inert extracts. All that had happened was that the weaker antibodies had disappeared leaving the strongest.

It is apparent from these experiments that biological activity is associated with several antigens. This might suggest that

the hormone was itself multiple, but these same antigens are common to both active and inert preparations. It would seem that the only possible interpretation is that the hormone is not itself an antigen but is carried on several substances probably protein and not themselves hormonal.

It is claimed that gonadotrophins are glycoproteins. Experiments with the diphenylamine colour reaction for glycoproteins shows that there is only a very crude correlation between the biological activity of a preparation and its glycoprotein content. Some inert preparations gave readings similar to those found in active extracts. Reference has already been made to the effect that with purification there is a loss of total activity and it has been found that there is also a loss of glycoprotein. Reference to the results given for the colour reaction will show that the benzoic acid preparations, Pregnyl and Gestyl possess no more glycoprotein

than crude extracts, although their total activity was many times greater. Indeed their activity per milligramme was several hundred times that of the other extracts. This discrepancy taken together with the other observations could indicate that the hormones are not even glycoproteins but some smaller molecule carried by several of the glycoproteins.

Conclusions.

1. The chemical process used in extraction of gonadotrophins from urines concentrates the antigens without altering their nature.

2. Nevertheless different methods seemed to extract different antigens in various strengths.

3. Benzoic acid extracts produced antisera with strong neutralisation and haemagglutinin activity. Tannic and Kaolin extracts produced antisera with precipitins, complement fixation antibodies, haemagglutinins and neutralisation. Neutralisation, however, was much less strong than with the benzoic antisera.

4. The precipitin reactions are apparently species specific. Complement fixation may be partly species specific while the haemagglutinin and neutralisation reactions are species non-specific.

5. Species specificity however does not appear to exclude hormone specificity altogether. The precipitate from precipitin reactions exhibited biological activity. The supernatant fluid still possessed neutralising

powers indicating that precipitation and neutralisation do not involve the same antibody.

6. There appear to be several antigens related to biological activity in varying degree.

7. In precipitin reactions the antibodies were associated with α , β globulins.

In reactions involving potent hormone extracts the antibodies were mainly of γ-globulin type.

8. Gel diffusion experiments demonstrated the multiplicity of antigens in all active extracts although each type of extract appeared to have a fairly constant number. Identity of reaction was demonstrated between the antigens of the various extracts.

9. The antigens which appeared to have biological activity were also found in inert extracts, suggesting that these antigens ought to be mere carriers of the hormonal activity.

10. Although small quantities of serum proteins were present in the hormone extracts these were not the antigens involved in the

reactions.

11. Glycoproteins were the main antigenic components and there were several in each extract.

12. Every method of purification removed part of these glycoproteins and with them some loss of total biological activity.

13. Benzoic acid and Sephadex gel filtration methods were most successful in purifying and concentrating the hormones. In doing so much of the glycoprotein content is removed.

14. Haemagglutinin reactions show less in the nature of cross reactions than any of the other tests. By dilution these cross reactions are minimised. For this reason this test used on urinary specimens may be employed for the purposes of pregnancy diagnosis.

15. Extracts inactive through long storage still give positive results with all the tests. The reactions are as strong as

those shown by active extracts, thus demonstrating that these reactions are not necessarily related to hormone activity.

16. The exact relationship of the glycoproteins to the biological properties of the gonadotrophic hormones is a matter for speculation. Two possibilities emerge. The hormone is itself a glycoprotein but present methods of purification are unable to separate it from other glycoproteins present in urine. On the other hand the hormone molecule may be a much smaller structure carried by these glycoproteins.

The results of our experiments tend to favour the second viewpoint.

APPENDIX

MATERIALS AND METHODS.

A. The Preparation of Antigens.

Kaolin Acetone Method.

Tannic Acid Method.

S.A.S. Method.

Sephadex Fractionation Method.

Preparation of the Crude Extracts.

Equipment.

Glass beaker of different capacities (100, 250, 600, 2000, 3000 ml.).

Mechanical stirrer (Tower's).

Measuring Cylinder 2000 ml.

pH meter (Direct reading E.I.L. Model 23A).

Water pump.

750 ml. Ehrlenmeyer flasks with stoppers.

Centrifuge for 100 ml. glasses and 100 ml. centrifuge tubes (M.S.E. Major).

Reagents.

Celite Powder.

Kaolin Powder (B.D.H.).

20% and 50% hydrochloric acid (Analar).

400% Sodium hydro oxide solution (Analar).

Acetone (Analar).

Ether.

Tricalcium phosphate prepared as described by Tiselius. (Loraine and Brown, 1954).

Phosphate buffered saline which is isotonic (pH. 7.2).

Sodium Chloride 34 g..

Anhydrous disodium hydrogen phosphate ($Na_2H\ PO_4$). 7.4 g..

Anhydrous Potassium dihydrogen phosphate (KH_2PO_4). 2.15 g..

Distilled water to 5000 ml.

Materials and Methods.

The kaolin adsorption with acetone precipitation as modified by Loraine and Brown (1956) was used, with a slight modification. The fresh urine was at first centrifuged at 2000 r.p.m. for 10 minutes after adding 2-3 gms. of celite to it. This, ensured the removal of all suspended matter, making the urine absolutely clean. Possibly some of the toxic substances were thus removed (Salhanick 1961).

The procedures of the technique were as follows:-

1. The centrifuged urine was measured in a graduated cylinder and its volume recorded. Then its pH was adjusted to pH 4 using 50 per cent hydrochloric acid.

2. Kaolin powder, one gram to each 100 ml. of the urine specimens, was added to the urine in a suitable beaker. The mixture was mechanically stirred for 30 minutes, then left overnight at 4°C.

3. Next morning the kaolin powder had settled to the bottom. The supernatant was sucked off, using a glass pipette connected to a tap vacuum pump.

4. An equal volume of distilled water was added to the remaining suspension. After thoroughly stirring, the suspension was brought up to pH 11-11.5 with 40% sodium hydroxide. The suspension was allowed to stand at room temperature for 30 minutes with occasional stirring.

5. At the end of 30 minutes, the suspension was centrifuged at 3000 r.p.m. for 10-15 minutes. The supernatant was kept this time. When it was intended to treat the extract with tricalcium phosphate, step 6 was performed. Otherwise the extract was treated as in 7.

6. The supernatant from 5 was brought to pH 8-8.5 with 50% HCl. Then its volume was measured in a graduated cylinder. Three ml. Tricalcium phosphate suspension were added to the extract, for each 100 ml. of the latter. The mixture was mechanically stirred for 15 minutes, then centrifuged at 3000 r.p.m. for 5-10 minutes. The supernatant was kept.

7. The extract solution was adjusted to pH 5-5.5 with 50 per cent HCl. Its volume was measured in a graduated cylinder. Five volumes of acetone were then added to the extract solution, and the mixture left overnight at 4°C.

8. Next day, the hormone extracts had precipitated to the bottom. The supernatant was sucked off. The powder was washed twice in absolute alcohol, and twice in ether. Each time the precipitate was stirred in the washing fluid with a glass rod, then centrifuged. After the last washing in ether

the precipitate was spread over the walls of the glass container with the aid of a glass rod which was left inside to prevent the loss of any powder. This spreading allowed the powder to dry at room temperature within ½ hour.

The powder was kept in a dessicator in a tightly capped glass container until it was required. It was then dissolved in 12.5 ml. of phosphate buffered saline (pH. 7.2). This solution was used as the neat in biological assays and serological tests.

Preparation of Purer Extracts.

1. Tannic acid method of Johnsen:(1958).

Equipment.

Two and 4 litre suction flasks with perforated stopper for evacuation.

Mechanical stirrer.

Buchner-funnel with porcelain perforated plate 95-100 cm. in diameter.

Waste-fluid flask with side tube for suction.

Cylinder glass with side tube for suction, with two marks, the first a 40 ml. mark, the second a 140 ml. mark.

Piece of thick perspex sheet, 3.5 x 10 cm. bent at an angle, 3.5 cm. from end (used for pouring fluid into a funnel without direct splash on filter-cake).

Vacuum water pump 65-70 Hg.

750 ml. Ehrlenmeyer flasks with a 600 ml. mark.

Centrifuge for 100 ml. glasses, 100 ml. centrifuge tubes.

pH meter (Direct Reading).

Reagents.

Glacial acetic acid, analar.

Sodium Chloride (analar).

Filter paper (hardened purified ashless paper (Balston Ltd. No. 541).

Hyflo-supercel and Celite powders (Johns-Manville, U.S.A.).

Tannic acid, analar (check for complete solubility in water).

Tannic acid solution, 10 per cent in distilled

water, prepared in stoppered flask shortly before use. Diluted solution (0.1 per cm.) is made by diluting some of the strong solution 1 in 100 with distilled water.

Ammonium hydroxide, concentrated, (25 per cent) analar (NH_4OH).

Ammonium acetate, analar (NH_4AC).

Absolute alcohol and 96 per cent alcohol.

Fifty three per cent ammonium acetate pH 11 (Stock solution 53-11-0).

Add 800-900 ml. conc. NH_4OH to 1 Kg. NH_4AC, placed in warm water and dissolve by stirring. Transfer to measuring cylinder and make up volume to 1900 ml. with conc. NH_4OH.

Ten per cent ammonium acetate pH 11 in 80 per cent alcohol (Solvent 10-11-80).

Ten per cent ammonium acetate pH 11 in 80 per cent alcohol (Solvent 10-11-80). This is prepared by mixing 1900 ml. solution 53-11-0 and 8100 ml. absolute alcohol in a 10 litre bottle.

Ten per cent ammonium acetate pH 11 in 40 per cent alcohol (Solvent 10-11-40). This is prepared by mixing 1900 ml. of solution 53-11-0, 3900 ml. distilled water, and 4200 ml. 96 per cent alcohol.

Phosphate buffered saline pH 7.2.

Procedures:

1. Acidification of Urine:

The urine sample was centrifuged with celite at 3000 rpm. for 10 minutes to make it quite clear. Successive portions of about 2.5 glacial acetic acid were added to the urine in a suitable beaker, until the pH was 4 ± 0.3.

2. Precipitation.

The urine volume was assessed approximately to nearest 1/4 - 1/2 litre 10 ± gm. sodium chloride was added per litre. Then 10-12 gm. hyflo-supercel were added, propeller inserted and mixture stirred. After 1 minute 20 ± 2 ml. of 10 per cent tannic acid were added and stirring continued

for 5 minutes. The propeller was removed and the flask was connected to vacuum. Stepwise evacuation with gently stroking was carried out, allowing the foam to settle. Then, suction was disconnected.

3. Formation of filter cake:

The Buchner-funnel was mounted and inserted in the waste-fluid flask with closely fitting filter paper. Two gms. (± 0.5) of hyflo-supercel suspended in 100 ml. water were poured into the funnel and allowed to settle.

When in the following procedure fluid was added to the funnel the piece of perspex sheet was held a little over the filter-cake and received the stream of fluid. In this way the filter cake was prevented from being stirred up by a splash of fluid.

The urine flask was shaken, then the funnel was filled with urine and the side-tube was connected to reduced vacuum (30 cm. water). Successive portions of urine were added

while this drained, the urine flask being shaken each time to keep the supercel suspended. The funnel was tapped a few times during this stage with a short piece of heavy rubber tube. Hyflo-supercel 2 ± 0.5 g. was added to the emptied urine flask, and 225 ± 25 ml. 0.1 per cent tannic acid solution. This suspension was poured into the funnel when the level of urine was near the cake. When the level again reached 0.3 - 0.4 ml. from the top of filter cake, the next step was immediately started without interruption of suction. At no stage was the cake allowed to run dry.

4. Differentiation.

225 ± 25 ml. 96 per cent alcohol was poured into the funnel and stirred gently over the cake. When the alcohol had nearly drained off, the same volume (225 ± 25) of solvent 10-11-80 was added and mixed by stirring. When the level of this solvent was near the cake the suction was disconnected.

5. Elution.

175 ± 25 ml. of solvent 10-11-40 were poured into the funnel and mixed over the cake. The funnel was then transferred to the cylinder glass, which was connected to reduced vacuum to allow 40 ml. to be suctioned through the cake. Then the suction was disconnected and the fluid allowed to drain slowly through the cake for 20 minutes. The rest was finally obtained (up to the mark 140 ml.) by suction.

6. Neutralisation and Precipitation:

The eluate was transferred to a 750 ml. flask. The sample was cooled in ice-water for 5 minutes. Then 10 ± 0.5 ml. glacial acetic acid was added during vigorous shaking. The volume was made up to 600 ± 50 ml. with absolute alcohol. The flask was rotated carefully to allow mixing, then allowed to stand overnight at 4°C.

7. Collection of precipitate:

Most of the clear supernatant was removed by suction next morning. The

remainder was transferred to a 100 ml. centrifuge tube, rinsing the flask with small portions of alcohol. It was then centrifuged, the supernatant discarded, the precipitate stirred with 60-80 ml. alcohol and centrifuged again. Two such alcohol washes were performed.

If the precipitate was abundant, washing with ether was done and the extract dried in air. Otherwise, without waiting for the precipitate to dry it was dissolved in 12.5 ml. phosphate buffer. This solution was used as the neat concentration for biological assays and serological tests.

II. Ammonium Sulphate fractionation of a Kaolin Extract.

Equipment.

Burette, to the tip of which a fine pasteur pipette was attached by a connection of rubber tubing.

Mechanical stirrer.

Glass beakers of capacity 250 marked at levels of 100 ml. and 150 ml.

Centrifuge for 250 ml. glasses.

Reagents.

0.1 M KH_2PO_4.

Phosphate buffered saline pH 7.2 (isotone).

Ammonium sulphate (analar).

A saturated solution (S.A.S.) prepared by dissolving 71 gm. of 4°C. or 77 gm. at 20°C. in 100 ml. of distilled water.

Procedures.

1. A solution extract of urine from a patient with Klinefelter Syndrome was prepared by the KA method. It weighed 320 mgms. of which 53.3 mgm. were dissolved in 12.5 borate buffer and kept aside as a control. The rest was stirred in 45 ml. 0.1 M KH_2PO_4 for one hour. The undissolved residue was removed after centrifugation and dissolved in 12.5 ml. of phosphate buffered saline. This constituted Fraction I.

2. The supernatant was poured in a 250 beaker after its volume had been made up to 45 ml. with KH_2PO_4. The beaker was placed on the

bottom shelf of a refrigerator at 4°C. Space was made to place over the beaker, the 100 ml. burette filled with saturated ammonium sulphate solution (S.A.S.).

Also a mechanical stirrer was placed beside the burette, so as to allow its spindle to pass along the burette and dip well into the solution in the beaker. The mouth of the capillary pipetted (attached to the burette) was kept under the surface of the solution.

3. The S.A.S. was added stepwise in 5 ml. portions. During the addition the solution was vigorously stirred. In this way the proteins were prevented from coming into contact with the S.A.S. before it was thoroughly mixed up. An interval of 10-15 minutes was allowed between the additions.

4. When the mixture reached the 100 ml. mark, bringing the S.A.S. saturation to 55 per cent, the stirring was continued for half an hour then stopped. The solution was

allowed to stand at 4°C. for 24 hours to complete the precipitation. The solution was then centrifuged and the precipitate (fraction 55) was collected and redissolved in 12.5 ml. of buffered saline.

5. The supernatant was returned to the marked beaker. Further addition of S.A.S. at 4°C. with continued stirring was carried out until the mark 150 ml. was reached. The stirrer was stopped and the solution allowed to stand for 24 hours at 4°C. Then the mixture was centrifuged, the precipitate collected and redissolved in 12.5 ml. saline. This constituted Fraction 70 or 70 per cent saturation.

6. The supernatant was measured in a measuring cylinder. The amount of ammonium sulphate powder to be added in order to achieve saturation was calculated. After mixing this amount with the solution it was kept at 4°C. for 24 hours.

The precipitate was collected after

centrifugation and constituted Fraction 100 or 100 per cent saturation. The supernatant was kept as Fraction 5.

III. Purification of Gonadotrophic Extracts on Sephadex G-25.

In 1959 Pharmacia Uppsala (Sweden) introduced a new material for use in a separation method called gel filtration. This material has been called Sephadex, of which three types are available according to the particle size. Sephadex g-25 has a coarse mesh (approx. 50-100 mesh). Its use was first described by Porath and Flodin (1959).

Several gonadotrophic extracts used in our work have been fractionated in gel filtration as an attempt at further purification. At the same time, an attempt was made to make the identification of the various fractions easy, by primarily labelling the extracts with a fluorochrome dye.

The method of ultra-rapid fluorescent labelling of proteins, as described by Rinderknecht (1962) was used.

Equipment.

Glass columns with sintered-glass discs.

Beakers of various sizes.

Reagents.

0.05 M Sodium Carbonate-bicarbonate buffer pH 8.5.

0.02 M Sodium phosphate buffer pH 6.5

Fluorescent isothiocyanate as 10 per cent in Celite (C grade).

California Corporation for Biochemical Research - Los Angeles).

Sephadex G-25: Pharmacia Uppsala (Sweden).

Procedure.

1. 2.5 gm. of sephadex were prepared by suspending them in distilled water, allowing them to settle down, then removing the supernatant with the suspended (too fine) particles.

This "slurry" was poured into a column half-filled with water, allowing the gel to

settle. Then the gel was washed with several column volumes of sodium phosphate buffer.

At no stage should the surface of the gel be disturbed, nor should it be allowed to become dry.

2. A mixture of 2 ml. of the hormone solution and 2 ml. of carbonate-bicarbonate buffer was shaken with 5-10 mg. of 10 per cent Fluorescen-Celite for approximately 3 minutes. The mixture was then centrifuged for 3 minutes.

3. The supernatant was allowed to flow into the column, taking care not to disturb the gel surface.

4. When the level of the labelled solution had reached 1-2 ml. above the surface of the gel the column was then developed with phosphate buffer.

Several bands were seen to separate with variation in the depth of colour as well as the hue.

Biological assay of the hormone extracts:

I. The House Uterus Test.

The technique used was that of Klinefelter (1943) as modified by Loraine and Brown (1956). The procedure was as follows:

1. Doubling dilutions of the hormone extract were made in normal saline. If the extract was expected to be highly active, the first dilution used in the test was the Neat which meant that each dilution was contained in 12.5 ml. If the extract was not expected to be highly active, the neat concentration was used. From it double dilutions were made in 6.25 ml. of saline. Dilutions up to 1/32 or 1/64 or higher were used according to the nature of the extract.

2. Immature female mice 21 days old (immediately after weaning) with body weights between 8 and 10 g. were used. These mice were bred from a colony originally obtained from the M.R.C. Endocrinological Research Unit in Edinburgh, and shown to have a fairly reliable response to unit doses of gonadotrophins.

3. Each mouse received 0.5 ml. of the

corresponding dilution intraperitoneally (Evans et al 1937) five times in the course of 3 days (total of 2.5 ml.). When the neat concentration was used, there was enough to use only two mice for each dilution (Johnson 1958). When the half dilution was started with, there was enough to inject 4-5 mice at each dilution. For each test a control batch was injected with normal saline instead of the hormone solution.

4. Autopsy was performed on the 5th. day. The uterus was dissected out, freed from blood vessels and connective tissue. They were blotted between layers of filter paper and weighed immediately on a precision balance (Oertling) with sensitivity of 0.2 mgm. per division and a capacity of 100 mgm. The mean uterus weight was calculated.

5. Expression of Results:

The highest dilution producing a mean uterine increase of at least 100 per

cent over the mean uterus weight of the control, was taken as the end point of activity. The total activity of the extract was calculated according to this end-part dilution as follows:

Neat dilution is equivalent to total activity of 5 m.u.u.

1/2 dilution is equivalent to 10 m.u.u.

1/4 dilution is equivalent to 20 m.u.u.

1/8 dilution is equivalent to 40 m.u.u. and so on.

Some modification of the route of injection was necessary when testing for the neutralising power of antihormones. These modifications will be referred to in the appropriate section.

APPENDIX (contd.).

B. The Serological Tests.

Precipitation Techniques.

Complement Fixation Techniques.

Haemoagglutination Techniques.

Gel Diffusion Techniques.

The Precipitin Tests.

Equipment.

Pasteur pipettes about 2-3 mm. diameter at their necks, sealed at their tip in flame.
Glass drippers (pasteur pipettes) diameter 1 mm. at their tip with rubber teats attached.
Tube racks and plasticine.
Incubator 37°C.
Centrifuge for micro-haematocrit head and the head attached.
Special plastic holder for centrifuging the micro-haematocrit capillaries (75 x 1 mm.).
Micro-haematocrit tubes marked at the level of 50 mm. (supplied by B.D.H.).
Black scale for measuring Beta precipitate.
Magnifying glass to view the precipitate for measurement.

Interfacial-ring Technique.

This was performed in the narrow neck of a Pasteur pipette sealed in flame at its tip.

One volume of antiserum was placed

in the pipette with a finer and longer pasteur pipette controlled by a rubber teat. The antigen solution was carefully superimposed in the same way.

The pasteur pipettes were placed in test tube racks, held in place with plasticine.

The first reading was taken after 1/2 hour at room temperature, the second after 2 hours, the third after standing overnight.

Then the pipette was flicked with the back of the index finger several times. This stroking augmented the reaction as well as allowed the precipitate to settle down to the bottom of the pipette. The precipitation was measured in millimetres with a ruler and for each mm. the result was reported as +.
The final reading was taken after standing overnight at room temperature.

Microprecipitation Technique (Immunocrit).

In order to conserve serum and antigen, and to use constant antigen and antibody concentration for quantitative comparison of

the reactions of different antigens, the reaction was performed in micro-haematocrit capillary tubes (Boyd 1956, ~~Findings of~~ McKean 1960, Haskell et al 1961, Chaney et al 1961).

One drop of undiluted serum was mixed with two drops of the antigen diluted 1/4 in phosphate buffered saline pH. 7.2. The mixture was thoroughly stirred on a clean dry glass plate, with the smoothened end of a glass rod. The mixture was then allowed to flow up a capillary tube to the 50 mm. mark. The end of the tube furthest from the fluid was carefully sealed in flame. The sealed tubes were held in special paper carriers. After ½ hour at 37°C. the capillaries were placed in special plastic holders and centrifuged on the haematocrit head for 5 minutes at 3000 r.p.m.

The precipitate at the tip of each capillary (if the reaction was positive) was measured in millimetres with a special scale

and magnifying glass.

A second reading was similarly taken after allowing the capillary tubes to stand at room temperature for two hours, a third after standing overnight.

The two techniques were used for different purposes.

1. To find out the optimal ratio of antigens and antibody, using a fixed concentration of antibody and rising dilutions of the antigen.

2. To estimate the precipitation titre of antiserum, using a fixed concentration of antigen (optimal concentration) against rising dilutions of antiserum.

Complement Fixation Tests.

Equipment.

0.1 ml. auto-zero high precision micro-pipettes (E.Mil Works). Test tubes 3 x ½ inch.

Cleaning of glass ware. For all serological tests the glassware was cleaned by repeated mixing in several changes of tap water and then changes of distilled water, drained by

immersion and shaking and dried in hot oven 100-110°C. No acid, alkali, soap detergent or spirit were used.

Complement.

Fresh guinea-pig serum was used. The blood was obtained by severing the large vessels of the neck over a 6 inch funnel, from which the blood was collected in a measuring cylinder. It was allowed to coagulate then stand overnight at 4°C. The complement was not used before 12 to 18 hours having passed from the time it was obtained. If not required immediately, it was divided in portions in tightly capped sterile universal containers and stored in the deep freeze. Pooled serum of several guinea pigs was used always.

2. Sheep red cells preserved in 10% formol and used within a week of its arrival (Burroughs Wellcome).

3. Haemolytic serum (Burroughs Wellcome) Glycerinated serum from horses injected with

sheep cells. It had a titre of 1/1000 to 1/2000.

Test Technique.

The Haemolytic System was prepared by making a 3% suspension of sheep red cells which had been washed in normal saline and centrifuged three times. To each 100 ml. of this suspension was added 0.2 ml. of the haemolytic serum. It was incubated at 37°C. for one hour, then kept at 4°C.

Titration of Complement.

A 1/10 dilution of the guinea pig serum was made. Seven tubes were used in which increasing quantities of the complement were added to 0.5 ml. of the haemolytic system. These quantities were 0.02, 0.025, 0.03, 0.035, 0.04, 0.045, 0.05 of the 1/10 dilution. The tubes were incubated at 37°C. in a water bath for one hour. The titre of the complement was read, as the first tube to show complete haemolysis.

The amount of complement in this tube

was taken as one M.H.D. (one minimal haemolytic dose).

The anti-complementary action of antigen and antiserum.

1. The antiserum was heated in a water bath at 55°C. for 30 minutes.

2. Two rows each of seven tubes were used. In one row 0.1 ml. of doubling dilutions of antiserum were pipetted. Similarly the antigen dilutions were placed in the second row.

3. A solution of complement was made up so as to contain 2 M.H.D. in 0.1 ml.

0.1 ml. of this solution was added to each of the tubes in the two rows in step 2.

The mixtures were shaken then incubated for one hour at 37°C.

4. To each tube was added 0.5 ml. of the haemolytic system. The tubes were shaken again and incubated at 37°C. for another hour.

The first tube to show complete haemolysis was chosen as the dilution of

antigen and antisera to be used in the test proper.

Test Proper.

Varying the complement.

1. A volume of 0.1 ml. of doubling dilutions of antigen, starting with the dilution chosen from the previous step, were pipetted in test tubes.

2. To each tube was added 0.1 ml. of the appropriate antiserum dilution.

3. Increasing amounts of M.H.D. were added to the tubes, each amount contained in 0.1 ml. of complement solution.

To the first tube was added 2½M.H.D. and 11 M.H.D. in the last tube, 10 tubes being used in each row.

4. Antigen Control.

To 0.1 ml. of the antigen dilution used in the first tube of the test was added 0.1 ml. of normal saline and 2 M.H.D. of complement.

5. Antiserum Control.

To 0.1 ml. of the antiserum dilution used in

the test was added 0.1 ml. of normal saline and 2 H.H.D.

All tubes were shaken and incubated at 37°C. for one hour.

6. To each tube was added 0.5 ml. of the haemolytic system. After shaking the tubes again, they were incubated at 37°C. for one hour. The strength of the reaction was read as the last tube to show complete haemolysis.

The readings were interpreted as follows:-

a. Negative (-) = Haemolysis complete.

b. Weakly positive (±) = Partial haemolysis.

c. Strongly positive (+) = No haemolysis.

If the antigen and serum control tubes did not show complete haemolysis, the test was repeated.

Titrating the antisera.

The same procedure as above was used except that the concentration of the antigen and complement were kept constant, while the dilution of antiserum was varied. The dilution of the

antiserum used in the first tube of the test was 1/10, thus double dilutions were made in subsequent tubes, using 8-10 tubes for each test up to a dilution of 1/640-1/2560).

Haemoagglutination Tests.

Two techniques were used:-

The first was the haemoagglutination test as used by Roitt et al, (1956), (Boyden's technique as modified by Stavitsky (1954). The second was the haemoagglutination inhibition tests as used by Wide and Gemzell (1960), Read and Stone (1958).

Equipment.

Glass test tubes 3 x 3/8 inch with round bottoms.

Test tube racks to hold the tubes.

Autoprecision 0.1 ml. micro pipettes. (E. Mil Works).

Ordinary glass pipettes (graduated) of different capacities (0.1 ml. to 10 ml.).

Reagents.

1. Blood cells: group O human cells obtained in citric acid-dextrose solution not used after 2-3 days.

2. Tannic acid: Analar Reagent tannic acid was used. It was made up into a solution of 1/20.000 (5 mgm/100 ml.) in buffered saline pH 7.2, freshly prepared immediately before use.

3. Buffered saline: pH 7.2 prepared as follows -

34 gm. NaCl.

7.4 gm. anhydrous Na_2HPO_4

2.15 gm. anhydrous KH_2PO_4

This volume was made up to 5 litres with distilled water. A few drops of toluene were added as a preservative.

Procedures.

Preparation of tanned red cells:

1. The red cells were washed and centrifuged three times in phosphate buffered saline.

2. A 4% suspension of the red cells was made

in buffered saline.

3. Equal volumes of the red cell suspension (4%) and the tannic acid solution (1/20,000) were mixed together and allowed to stand at room temperature for 30 minutes.

4. The cells were then centrifuged at 500 r.p.m. for 5 minutes, and washed once in buffered saline. They were resuspended in buffered saline to make a 2% suspension.

Sensitisation of the Red cells with Antigen.

1. An equal volume of the 2% suspension of tanned red cells was mixed with an equal volume of the antigen solution. The mixture was left at room temperature for one hour with occasional shaking.

2. The mixture was then centrifuged gently (500 r.p.m. for 5 minutes), and subsequently the sensitised cells were washed carefully three times with buffered saline.

3. The sensitised cells were finally suspended in 1% suspension in buffered saline.

Antiserum.

The antiserum was heated at 55° for 30 minutes to destroy the complement (C_1) allowing the complement fraction (C_4) involved in haemoagglutination to work strongly.

Dilution of the antiserum were made in buffered saline, starting with a 1/10 dilution. The dilutions used were 1/10, 1/20, 1/50, 1/100, 1/250, 1/500, 1/1000, 1/2500, 1/5000, 1/10,000.

Test Proper.

The Haemoagglutination Techniques.

1. 0.4 ml. of each serum dilution was pipetted in the appropriate tube.
2. The control tube received 0.2 ml. of the 1/10 serum dilution plus 0.2 ml. of the concentrated solution of the antigen tested.
3. To each tube was added 0.1 ml. of the 1% suspension of antigen-sensitised red cells.

The tubes were shaken, then allowed to stand at room temperature for 2 hours when

the first reading of the test was recorded.

The tubes were left at 4°C. overnight and a second reading taken.

The control in this reaction was an inhibition control, i.e. there should be no agglutination in the control tube.

Haemoagglutination Inhibition.

The reagents used in the previous test were also used in this one. The procedures were the same in both, except for the nature of the mixture in the tubes.

1. A constant dilution of antiserum (1/10 or 1/20) was used in all tubes. 0.2 of this dilution was put in each tube.
2. Rising dilutions of the antigen were made in buffered saline. Of each dilution 0.2 ml. was added to the antiserum in one of the tubes. The antigen dilutions used were - 1/10, 1/20, 1/40, 1/80, 1/160, 1/320, 1/640, 1/1280, 1/2560, 1/5120.
3. A control tube contained 0.2 buffered saline plus 0.2 of the first antigen dilution

used, but no antiserum.

4. 0.1 ml. of the sensitised red cells suspension (1%) was added to each tube.

The tubes were shaken, allowed to stand at room temperature for two hours and the first reading recorded. They were kept at 4°C. overnight when the second reading was made.

In this case the control should be negative, i.e. shows no agglutination while no agglutination in the tubes of the test is a positive result.

Agglutination Patterns.

1. Complete agglutination: smooth mat on the bottom of the tube. The edge may be somewhat ragged or folded.

2. Partial agglutination: a narrow rim of cells around the edge of a smooth mat.

3. No agglutination: the cells form a button in the centre of the tube.

4. Collapsed pattern: a crumpled mass of cells due to non-specific factors.

The Gel Diffusion Technique.

Equipment.

Four inch petri dishes sterilised in the hot air oven at 160°C. for 60 minutes.

Metal cork borers of different sizes.

Surgical scalpel with blade.

Incubator 56°C. - Incubator 37°C.

Empty biscuit tins.

Reagents.

Difco agar (purified Bacto-agar Difco).

Sodium chloride solution 0.85%.

Thiomersalate powder.

Nigrosine stain (Gurr T).

Naphthalene Black B10 stain (Gurr).

Methyl alcohol (acetone free).

Glacial acetic acid (Analar).

Preparation of Plates. (Cruickshank & Currie, 1958).

1. A solution of 2% Difco agar in 0.85% saline solution containing 1/10,000 thiomersalate was autoclaved for 30 minutes.

2. If not used immediately, the solution

was divided into 10 ml. aliquots, each aliquot kept in a sterile universal container and allowed to solidify. When required, each universal was placed in boiling water until the agar melted, then the agar was poured in a petri dish and allowed to solidify. Thus repeated heating of the agar before use was avoided (Matthews 1958).

3. Some of the plates used, (when weak reactions were expected) were prepared by pouring 1 ml. of 0.001 nigrosine in normal saline in a universal containing the melted agar before it was poured out. (Butt et al 1961).

4. The plates were dried at 37°C. for 30 minutes.

5. With cork-borers a central cup and several peripheral cups were cut out in the agar sheet. The number of the peripheral cups varied from 4 to 8. According to the size of the central cup (8 mm. to 10 mm.) the size of the peripheral cups (6 mm. to 8 mm.) varied.

Also, if the number of the peripheral cup was more than 6, the distance between the circumference was 1.5 cm. otherwise the distance was 1 cm.

Procedures: (Cruickshank & Currie 1958, Henry & Van Dyke, 1958).

1. Undiluted antiserum was placed in the central cup till it was full. The peripheral cups were filled with the different antigens to be tested. Duplicate plates were always made.

2. The plates were kept in empty biscuit tins at room temperature. They were observed every 2 to 3 days. When the cups became empty, they were filled only once with normal saline.

Usually maximum development of the precipitin bands required 5-10 days. At this time the plates were photographed. Then they were stored at 4oC to study the effect on time on the bands of precipitation.

3. Some of the plates were preserved

permanently according to the method used by Hutchison (1962) which was as follows:-

a. Overnight washing of plates in running tap water.

b. Floating the gel sheets onto clean glass plates then drying them at 56°C. for 2 hours.

4. Staining the dried film of gel with naphthalene black or nigrosine until the lines were suitably stained. Then, differentiating in the appropriate solids cleared the back-ground from the stains.

Interpretation of the Results.

The bands of precipitation which appeared between the antigen and antisera cups were observed for the following -

1. Time of appearance.

2. Number and relative intensities.

3. Identity or non-identity of the bands of reaction which showed as follows: (Ouchterlony 1949, Henry and Van Dyke 1958, Cruickshank and Currie 1958).

a. When precipitin bands of different

antigenic extracts joined or approached as if to tend to join (Reaction of identity) this indicated identical antigenic components.

b. When the precipitin bands crossed each other (reaction of non-identity) the antigenic extracts contained non-identical components.

c. If part of one band, after joining the corresponding band extends beyond the point of junction (reaction of partial identity) this indicates components which are chemically identical but only partly identical antigenically.

Photography.

The precipitin lines in the petri-dishes were photographed as follows:

1. The petri-dish (without its lid) was placed in the central hole of the black cardboard sheet, on a table, facing forward in a dark room.

2. The two flood lamps were placed 2 feet behind the plate, and at 45° angle to the back of the cardboard sheet. A sheet of

black perspex was held further back to provide a black background.

3. The exposure time and aperture opening were estimated with an exposure meter. Two exposures were made for each plate using a 35 mm. film.

APPENDIX (contd.).

C. Miscellaneous Techniques Used.

Vaginal Smears.

Serum Electrophoresis.

Fractionation of Serum Proteins.

Colorimetry of Glycoproteins.

Other Techniques.

A. Electrophoresis.

Paper electrophoresis of antisera before and after reacting with different antigens was required to clarify some points. Also electrophoresis of some of the urinary extracts used as antigens was done.

Equipment.

1. Whatman 3 mm. paper strips cut into 2.9 x 30 cm. pieces. (Flynn and DeMayo 1951)
2. A vertical tank (Shandon).
3. A unit for holding the paper strips in the vertical electrophoresis tank, with a glass rod to support the strips at their middle.
4. A power unit (Shandon Scientific Co.Ltd., 6 Cromwell Place, London. S.W.7.).
5. Hot air oven of a temperature 110-120°C.

Reagents.

Electrophoresis Buffer.

Barbital Buffer, pH 8.6 ionic strength 0.075.

Naphthalene Block B12 saturated solution in methanol-acetic acid solvent (1 gm. per 100 ml.).

Solvent (methanol acetic) for washing.

Procedure.

Application of serum:

1. 0.06 ml. of absorbed sera or control sera (all being a 1 in 3 dilution of original serum) was applied with a pipette to the middle of the paper strip, with intermittent drying in air.

2. Each end of the paper was dipped in buffer separately allowing the buffer to come within 1-2 cms. from the origin.

3. The strips were placed in position so that the point of application (middle) was supported by the glass rod. Each end was dipped in a tank containing the buffer. The buffer was allowed to rise up both sides by capillary action to the origin. Then the strips were tensioned across the rod.

4. Separation was carried out with a

constant current of 8 amperes for four strips (Voltage of 100-120) for 16 hours at room temperature.

The strips were then removed, hung on a rod in the hot air oven and dried at 110-120° for 30 minutes.

Staining.

The strips were dyed for 10 minutes in Naphthalene Black 12B, then washed in successive baths of fresh solvent until the background was light blue.

They were finally washed in water and dried at 110-120°C.

Scanning.

Because of the problem of dye uptake, the strips were always compared with a control run in same test. The dyed strips were scanned by passing them in an automatic scanner of the recording reflector densitometer type (chromograph, Joyce & Loebel). A direct graph was obtained for each strip.

B. Vaginal Cytology.

For many years Papanicolaou studied the cellular content of the vaginal smear both in laboratory animals and in the woman. The work related to rodents is well known (Stockard & Papanicolaou 1917), and has been of great importance in advancing the knowledge of endocrine physiology. Some of the antisera used in this study were tested for their power to inhibit endogenous gonadotrophic hormones in rodents. Adult female rats were injected intraperitoneally with the antiserum and the daily changes in the vaginal smear were studied over a period of injection which lasted two weeks. Each rat was first checked by daily smearing for five days before starting the injections. A full cycle should occur in four to five days in the rat. One rat received antigestyl serum, one received antipregnyl serum and one anti-F.S.H. serum (anti-Klinefelter extract serum). Each rat

received 1 ml. of the corresponding serum intraperitoneally once daily for two weeks.

Technique of taking the smear.

A piece of clean non-absorbent cotton wool, wound round the end of a wooden swab stick, was pushed up the vagina to the vault and rotated through three complete circles (Spira and McRae 1960). The moist swab was then rubbed on a clear dry glass slide to transfer as much of the smear as possible on to the slide. The slide was immediately plunged into a solution of equal parts of 95% alcohol and ether in a coplin jar.

Staining of the Smear. (Papanicolaou 1942).

Equipment.

1. Staining counter.
2. Covered staining jars (each of a capacity of 400 to 600 ml.).
3. Slide carrier and slide forceps.
4. Interval timer, funnel, filter papers and

gauze.

5. Mounting media (Depex) and coverslips.

Reagents.

Harris Haematoxylin. } Ortho Pharmaceuticals.
OG.6. }
EA.36 }

Hydrochloric acid.

Lithium Carbonate.

Ethyl alcohol (Analar).

Xylene (Analar).

Distilled Water.

Procedures.

1. Hydration of Smears.

They were brought down to distilled water through descending grades of alcohol (70%, 50%, 30%) dipping them 10 times in each grade.

2. Smears were stained for 1-3 minutes in Harris Hx and excess stain washed off in tap water.

3. The smears were then differentiated in 0.25% HCl for 3-5 dips, then again washed in water for 1 minute.

4. They were dipped in Lithium carbonate

for 10 times to neutralise the acid, then washed in water for ½ minute.

5. The smears were then dehydrated by 10 dips in ascending grades of alcohol (30%, 50%, 70%, 95%).

6. The smears were now stained for 2-3 minutes in OG.6 and rinsed twice in two changes of 95% alcohol.

7. The smears were stained for 2-3 minutes in EA.36 and were rinsed in two changes of 95% alcohol. They were finally dehydrated by 10 dips in each of two changes of absolute alcohol.

8. They were then cleaned in two changes of xylene for a few minutes, then mounted in synthetic resin media (Depex) and covered with clean cover slips.

C. Salt Precipitation of γ -globulin.

Several workers have used ammonium sulphate to precipitate the γ -globulin fraction of the serum (Wolfson et al 1948, De la Huerga & Pepper 1950). The method used was that

of Wolfson et al.

Equipment.

Centrifuge for 40 ml. tube and 40 ml. glass tube.

Viskase dialysis tubing (½ inch diam.).

(Scientific Hospital Suppliers Ltd.).

Reagents.

Sodium chloride-ammonium sulphate solution prepared as follows:-

193 gm. ammonium sulphate (Analar) were dissolved in 500 ml. distilled water. Then 40 gm. of sodium chloride (Analar) were added and dissolved. The volume was made up to one litre with distilled water and kept at room temperature.

Technique.

1. 38.4 ml. of this sodium chloride-ammonium sulphate solution was poured into a 40 ml. centrifuge tube. A volume of 1.6 ml. of serum was layered on top and mixing was done by slow careful inversion until the turbidity appeared to have reached a

maximum.

2. The tube was corked and cooled in a beaker containing ice cubes for 5 minutes then centrifuged for 30 minutes at 2500 r.p.m.. If the supernatant fluid was still not quite clear, the tube was cooled for 5 minutes, and centrifuged again for a few minutes.

3. The supernatant was poured off carefully. The precipitate was dissolved in 0.5 ml. of distilled water.

4. The γ-globulin solution obtained in (3) was transferred by a glass dropper into a piece of dialysis tubing which had been tied at its end. The tube was then tied above the solution allowing an empty space twice as much as that occupied by the solution. This Viskase (sausage) was dipped in a large glass tank containing distilled water. It was prevented from floating by tying both ends to a suitable frame of glass rod. The distilled water

was changed frequently in the tank.

Dialysis was continued for 24 hours.

The D.P.A. Test for quantitation of Glycoproteins.

Equipment.

Reaction tubes: glass stoppered tubes (6 x 5/8) inch with a central hole in stoppers to permit equilibration of pressure with the atmosphere.

Electrically heated water bath with special rack to hold the reaction tubes.

EEL Colorimeter.

Spectrum green filter (EEL 624).

Reagents.

Diphenylamine powder (Analar).

Glacial acetic acid (Analar).

Concentrated sulphuric acid H_2SO_4 (Analar).

The diphenylamine reagent was prepared as follows:

1 gm. diphenylamine.

90 ml. Glacial acetic acid.

10 ml. Conc. H_2SO_4.

It was kept in a well stoppered dark glass bottle.

Procedures.

1. Of each of the doubling dilutions of the extract to be tested, 2.5 ml. were pipetted in a reaction tube. Then 2.5 ml. of the D.P.A. reagent were added and the tube stoppered. A blank of 2.5 normal saline was similarly treated.

2. The reaction tubes were placed in the rack of the water bath which was filled with water to a suitable level, and which should be at the boiling point when the tubes were placed into it. The reaction was allowed to take place at 100°C. for 30 minutes. Then the reaction tubes were removed from the bath and allowed to cool down.

3. A purple colour developed in mixtures of most extracts used. Sometimes a greenish colour occurred. This was due to nitrates contaminating the reagent or one of the solvents and meant that fresh solutions of all reagents had to be prepared.

4. The colour was read against the blank

in an EEL colorimeter using 3 ml. tube and an adaptor to fit them. If the reading was 100 or more it was disregarded, and the colour of a higher dilution was considered.

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